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Efeito da salinidade no crescimento, nutrição e compostos secundários de *Diplotaxis tenuifolia*

Salt effects on growth, nutrient and secondary compound contents of *Diplotaxis tenuifolia*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Dr.Conceição Santos, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro e do Dr. Jelte Rozema Professor da Vrije Universiteit de Amesterdão, Holanda.

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Palavras-chave

Diplotaxis tenuifolia, balanço de nutrientes, crescimento vegetal, polifenóis, respostas fisiológicas, queracitina, stress salino

resumo

A salinidade do solo é considerada, a nível mundial, como o principal factor abiótico de stress agrícola. Este facto levou ao desenvolvimento de diversos projectos em tolerância salina com o intuito de melhorar as culturas tradicionais e descobrir novas plantas passíveis de crescer nesses solos. Este trabalho centra-se nas respostas de crescimento de *Diplotaxis tenuifolia* L quando cultivada sob condições de stress salino. O conhecimento do grau de tolerância salino desta planta é de grande interesse para a agricultura, uma vez que existem observações da ocorrência natural desta planta em zonas sujeitas a stress salino. As plantas foram cultivadas num gradiente de solução nutritiva com as concentrações de 0, 50, 100, 200 e 300 mM NaCl. Em todos os tratamentos as plantas não só sobreviveram, como cresceram e floriram. No entanto, notaram-se alguns sinais inibitórios de crescimento para os dois valores de salinidade mais elevados. O peso seco e a área foliar foram medidos e a o crescimento relativo (RGR), bem como os seus componentes, rácio foliar unitário (Unit Leaf Ratio – ULR, comummente designado por NAR) e rácio de área foliar (Leaf Area Ratio – LAR), foram calculados. A expansão da área foliar decresceu com o aumento de salinidade. A produtividade fisiológica máxima (ULR) foi atingida nos 100 Mm NaCl. LAR foi provavelmente o maior responsável pelo ligeiro aumento nos valores de RGR, já que o ULR se manteve relativamente constante. No entanto o crescimento relativo manteve-se constante até 100 mM NaCl não apresentando diferenças significativas. A acumulação de iões como Ca^{2+}, Mg^{2+}, K^{+}, decresceu com o aumento da salinidade, ocorrendo comportamento inverso com o Na^{+}, que atingiu valores bastante elevados nas salinidades mais altas. O rácio Na/K aumentou significativamente com o aumento da salinidade, sendo de notar que nas folhas velhas se obtiveram valores muito mais baixos que nas folhas novas, o que é indicativo da compartimentação do Na^{+} para as folhas mais velhas. O conteúdo total de polifenóis e do flavonóide queracitina foi máximo nos 50 mM NaCl. O conteúdo em azoto decresceu com o aumento da salinidade mas não tão acentuadamente como nos restantes iões (Ca^{2+}, Mg^{2+}, K^{+}). Os resultados revelaram um crescimento óptimo desta planta a salinidades moderadas, com o máximo de produtividade (peso seco) a ser atingido a 100 mM NaCl. De acordo com o sistema de classificação usado, *Diplotaxis tenuifolia* foi classificada como uma espécie tolerante ao sal até um valor de salinidade máximo de 100 mM. Os resultados deste trabalho servirão de ajuda a programas de implementação de culturas de agricultura salina que visam uma maior sustentabilidade agrícola.

Keywords *Diplotaxis tenuifolia*, nutrient imbalances, plant growth, phenolics, physiological responses, quercetin, salt stress

abstract Soil salinity is considered a major abiotic stress in plant agriculture worldwide. This has led to several research projects into salt tolerance with the aim of improving crop plants. This work focuses on the growth responses of *Diplotaxis tenuifolia* L. when grown under salt stress conditions. Having been found growing in salt exposed areas this plant's degree of salt tolerance is of interest to saline agriculture. Plants were grown in water culture with the concentrations 0, 50, 100, 200 and 300 mM of NaCl. Plants survived, grew and reproduced in all salinity treatments. However, signs of growth inhibition were noticed for the highest salt concentrations. Plant dry matter content and leaf area were successfully measured and relative growth rate (RGR) and its components, Unit Leaf Rate (ULR, usually known as NAR) and Leaf Area Rate (LAR) were calculated. Expansion of plant leaf area reduced with increasing salinity, with the highest physiological plant productivity (ULR) obtained at 100 mM NaCL. The slight increase in RGR values was probably due to higher values of LAR, while values for ULR presented little variation. Nonetheless RGR was very constant for the first salinities presenting no significant variation. Accumulation of ions such as Ca^{2+}, Mg^{2+}, K^{+} decreased with increasing salinity and Na^{+} increased to very high values. Na/K ratio increased significantly with increasing salinity; however old leaves showed much lower values for this ratio than the new leaves, which may indicate a compartmentation of Na^{+} towards the old leaves. Total polyphenol content and quercitine was maximum at 50 mM NaCl. Nitrogen content decreased with increasing salinity although not as drastically as other ions (Ca^{2+}, Mg^{2+}, K^{+}). Results showed optimal growth at moderate salinities and maximum productivity (dry weight) at 100 mM. According to literature agricultural crops classification, *D. tenuifolia* is a tolerant species with a threshold value of 100 mM. The findings of this work will help the agricultural sustainability programs in the selection for halophytic species for their economical potential.

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Abreviations

DW – Dry Weight
EC – Electric Conductivity
FW – Fresh Weight
NAR – Net Assimilation Ratio
NS – Nutrient Solution
RGR – Relative Growth Rate
SLA – Specific Leaf Area

Introduction

"Even though agriculture is the first human activity, after years of evolution it is still not able to feed the global population (Galvani 2007)".

If one asks what are the most urgent problems man faces for the next decades, several authors will answer: freshwater and food (Galvani 2007, Khan and Duke 2001, Shah et al. 2000, Yensen 2006).

Freshwater is a scarce resource and, according to Grid-Arendal, an official United Nations Environment Program (UNEP), of the total water volume only 1% is freshwater, 1% is brackish and 98% is seawater (Grid/Arendal 2008). When looking at these numbers it becomes obvious that seawater is an abundant resource to be used and, therefore, reduce the amount of freshwater used in conventional agriculture, which comprises 70% of all freshwater resources used by man (Hodges et al. 1993). Also, with the current growth in the world's population there will be approximately eight billion people in 2025 (UNPF 2008), this means that food requirements will increase 20% in developed countries and 60% in developing countries. In addition, a considerable expansion of the production of non-food items is needed, such as fuel, fibbers, fodder, and other agro-outputs (Galvani 2007).

Therefore, conventional agriculture alone can no longer suppress the coming needs, a fact that pressures society to find and use other natural resources. The most obvious solution to this problem is to resort to saline agriculture. This one uses unexplored resources like the huge amount of saline and salinized water available and the already existing salinized soils (Yensen 2006), considered nowadays as waste land. The idea of using seawater for crop production along coastal deserts has been proposed over the past 30 years (Boyko 1966, Epstein et al. 1980, Glenn et al. 1995, Glenn et al. 1997), but it's only now that society is turning its attention that way and only in the latter part of the 20th century that serious efforts began to be developed. Yensen (2006) stated that "*The 21st century will likely be the century for halophyte agriculture expansion*" and this concept was supported by many other authors (Choukr-Allah 1993, Khan and Duke 2001, Koyro et al. 2006).

RESOURCES FOR SALINE AGRICULTURE

Soil availability

Salinity is a global problem that occurs in approximately 1 billion hectares all over the world (Choukr-Allah 1993, Flowers 2004, Yensen 2006). In the Mediterranean basin alone it comprises around 80 million hectares – more than 40% of the total area (Choukr-Allah 1993, Nedjimi et al. 2006).

Although soil salinity is increasing nowadays, it exists since long ages, even before man (Zhu 2001) and the places most affected by salinization are the arid and semi-arid areas (Bernstein 1975, FAO 2008) and this may include many regions in the world, but particularly the Mediterranean Basin (Choukr-Allah 1993), Australia, Central Asia, the Middle East and Northern Africa (FAO 2008, Yensen 2006). About 23% of the world's 1.5x10^{9} ha of cultivated

land is saline and 37% is alkaline (Khan and Duke 2001). According to some estimates, about 200 million hectares of new plantations will be needed in the next thirty years in order to feed the growing populations of the tropical and subtropical countries. However in these areas only 93 million hectares are available for farming (Galvani 2007). The situation is being made worse not only by the population increase but also by the resulting deterioration of the soils (Choukr-Allah 1993). Contributing to the deterioration of the soils are among other factors the unsustainable irrigation practices, the growing pressure from cattle on pastoral zones, deforestation, etc. Salinized soils tend to increase every passing day (Nedjimi et al. 2006), therefore an effort has to be made in order to face these salty areas as opportunities for improving life quality and not as problems (Yensen 2006).

Overall, desert/arid soils, salt marshes and/or salt water are regarded as highly disadvantageous to conventional crops, but they may present many advantages to halophytic plantations. For example, of the thirteen mineral nutrients used by plants, eleven are present in salt water in concentrations suitable for farming (Galvani 2007). According to (Yensen 2006) it is possible, with the development of halotolerant and halophytic crops, to produce twice the amount of food produced today. Selection of halotolerant plants is a strategic alternative for a sustainable agriculture defended by several authors (e.g. Nedjimi et al. 2006).

Saline water

Saline water for halophyte crop irrigation can have many origins: seawater; brackish water from estuaries, salinized phreatic sheets, drainage water from other plantations irrigation; drainage water from humanized areas – e.g. sewage (Grieve and Suarez 1997, Yensen 2006), aquaculture waste water (Porto et al. 2006). Therefore there is a tremendous availability of saline water for halophyte production under sustainable regimes, and in some cases this activity may be performed in combination with other industrial activities. However, the use of saline water for irrigation may increase soil salinity (OASE 2005), so this strategy (and the selection of crops) must be carefully planned and adequate technology for soil preservation is recommendable (Galvani 2007).

Trees and plant species diversity

In a global level, halotolerant and halophyte plant diversity is huge (Yensen 2006). Around 6000 species of terrestrial and tidal halophytes in the world, and 700 species only in the Mediterranean climate area (Choukr-Allah 1993), have qualities that make them suitable for growing in saline environments (OASE 2005). A more precise estimate of the total number of halotolerant and halophyte species/genotypes is complicated since the plant diversity lists available today do not make this distinction (Yensen 2006). The list of species/genotypes checked so far for potential utilization is therefore small in comparison to the salinity tolerant species available.

The floristic composition of most plant communities occurring in salt marshes is low when compared with the nearby vegetation of fresh water habitats. The lower diversity of species in salt marshes and coastal deserts is correlated with the fact that many of the vascular plants, either terrestrial or aquatic, do not possess the necessary adaptations to salt tolerance (Ungar 1991).

CROPPING POTENTIALITIES

Cropping potentialities for halophytes goes as far as the possibility to suppress the needs of an entire community on food, shelter, fuel and ornamentals (Yensen 2006). The Seawater Foundation farm with the project "Greening Eritrea" is a good example of transforming desert-like area into useful land, completely irrigated with seawater and producing from fish to flour and timber (possible to view at www.seawaterfoundation.org). Salt-tolerant plants can be used to produce economically important materials such as essential oils, flavours, fragrances, gums, resins, oils, pharmaceuticals, and fibbers (Galvani 2007).

Cropping halophytes may contribute to human well-being through many ways including: (1) protection and conservation of soil and water resources; (2) improvement of soil structure and fertility; (3) providing habitat for wildlife; (4) creation of recreation space for sport and leisure; (5) source of biomass for the production of biodiesel, which has become so attractive recently; (6) improvement and protection of the environment from pollution such as sediment, wind-blowing soil, municipal and farm wastes, and some toxic substances (Barnes and Baylor 1995, Zhang and Wang 2007).

Food

Appropriate salt-tolerant plants currently growing in saline soil or water can be domesticated and their seeds, fruits, roots, or foliage used as food (OIA 1990). In some European countries plants like *Diplotaxis* and *Salicornia* are eaten boiled or in salads and one can also find it served in many high rank restaurants. Even, for example for *Salicornia*, when the plant itself is too salty for direct consumption it is possible to extract for example the leaf protein and use it to make fortified spaghetti (Galvani 2007).

Forage

Halophytes are a traditional source of animal nutrition, even though they may present problems of high salt content, low energy content and low palatability compared to conventional feeds (Glenn et al. 1993).

Atriplex sp., *Tamarix* sp. or *Nitraria retusa* are examples of grasses grown in marginal agricultural areas (Zhang and Wang 2007), and almost always are over grazed and disappear rapidly due to high grazing pressure of most animal species. These are grasses that can provide good fodder for livestock and wildlife, but as individual forage materials, have little prospect because long feeding periods are known to have adverse effects on browsing animals. So, mixing halophyte forage with feed materials that are rich in protein or energy can significantly improve the feed nutritional value. Shaer (2006) reported that a mix feed of dry grass and green *Atriplex* materials increases goat weight in Pakistan.

Grains and oilseeds

Many seed-bearing halophytes have an interesting characteristic, although they may have significantly greater levels of salt in stems, branches, and leaves than conventional plants, their seeds are relatively salt-free (OIA 1990). This means that from the almost fifty species of seed bearing halophytes and halotolerant plants growing in the world (OIA 1990), we can use some

of them to produce very much needed products like biofuel, vegetable oil, feeding complements, etc.

Salicornia bigevolli is the best studied halophyte species until today. It is an annual, succulent plant that is grown for its oilseed as well as straw. The oilseed is low in salt being is extracted by conventional milling procedures. Although the oil is apparently a valuable edible oil for human use it can also be used in animal diets. The seed meal left after pressing out most of the oil contains approximately 33-34% crude protein. Animals fed with *Salicornia bigevolli* have equivalent growth rates to those fed with equal amounts of conventional forages like for example wheat straw and alfalfa (Glenn et al. 1993).

Halophytes explored in cultures using saline water (Shaer 2006):

The use of halophytes in commercial cultures/exploitation, though still limited, is already being used for some species. Some examples are listed below:

Halophytes for food such as *Aster tripolium* (salt aster), *Salicornia* sp., *Avicennia marina* and *A. germinant*;
Halophytes for wood such as *Tamarix* spp. and mangroves;
Halophytes grown for chemicals: a variety of halophytes are collected for health and beauty products purposesants can cover barren soils in a short time, e.g., *Batis maritima*, *Sesuvium portulacastrum* and *Atriplex* spp.;
Ornamental halophytes such as *Limoniastrum monopetalum* and *Aster tripolium*;
Environmental protection: Many halophytic species are used for coastline protection such as *Spartina alterniflora*, *Spartina maritima* and *Avicennia marina* (Callaway and Zedler 2004, Shaer 2006).

WHAT NEEDS TO BE DONE NEXT?

A germplasm collection, which has been proposed since 1999 by Böer and Ghais, can assist worldwide when halophytes are required for habitat restoration, as well as for biosaline agricultural research (Böer 2006, Yensen 2006). There is information for more than 100 species about their salt tolerance; however discrepancies and inconsistencies exist in some of the information due to differences in cultivars, environments, and experimental conditions. For a great number of species little or no useful information exists and there is an obvious need for research. As proposed by Lieth et al. (2002), systematic researches should be initiated to get seeds from all plants to be tested about dormancy and germination requirements, juvenile and adult growth, and fruiting peculiarities.

Diplotaxis tenuifolia, why this species?

In the pursuit of new edible cropping species that can cope with salt stress *Diplotaxis tenuifolia* was chosen due to its natural occurrence in areas within the sea's influence in spring tides, and daily salt spray, thus being suspected to be a halophyte plant. As stated above the use of *Diplotaxis* species in cultures using salt water has large potential, as food, forage, etc. However, and despite it is being already grown as a conventional crop, little is known about its performance (e.g. growth, yield) under salt water conditions, and the potential of using salt

water irrigation systems in *Diplotaxis*' large scale production. We describe here some of this genus characteristics.

Distribution, Taxonomy and Morphology

The genus *Diplotaxis* L. (DC) is originally from the Mediterranean region (D'Antuono et al. 2008) however nowadays it can be found growing more or less, all over the world. It can be found growing in different kinds of soils but preferably calcareous. It occurs in fields, along roadsides, waste places, beaches and rock crevices (Bianco 1996).

Commonly known as "wild rocket" or also "perennial Wall-rocket", the species *Diplotaxis tenuifolia* is "not protected" according to Ruggiero (2007) from the Global Biodiversity Information Facility. Although the name "rocket" includes several taxa belonging to the genera *Eruca* and *Diplotaxis*, cultivated and consumed rocket corresponds mainly to *Eruca vesicaria* subsp. *sativa* (Miller) Thell and only after *Diplotaxis tenuifolia* (L.) DC. These two species are morphologically similar, however *Diplotaxis tenuifolia* (from the Greek words 'diplos' = double and 'taxis' = row, referring to the seeds placed in double rows in the siliqua) can be distinguished by a perennial and suffruticose habit at its base and its leaves entire to pinnatifid have a piquant sometimes pungent flavour a bit stronger than that of *Eruca sativa*.

Taxonomy:
Kingdom Plantae
Division Magnoliophyta
Class Magnoliopsida
Subclass Dilleniidae
Order Capparales
Family Brassicaceae - mustards
Genus *Diplotaxis* DC - wallrocket
Species *Diplotaxis tenuifolia* (L.) DC - perennial wallrocket

The Brassicaceae family, in which wild rocket is included, plays a major role in worldwide vegetable production and consumption, ranking second after Solanaceae, e.g., potatoes and tomatoes. Within the vegetables, *Brassica* species are of high nutritional and health-promoting value. It's a large family of plants including major vegetable crops such as broccoli and cabbage and also salad and herb species such as *Eruca sativa* L., *Diplotaxis erucoides* L. (wall rocket), and *Bunias orientalis* L. (Turkish rocket; Turkish warty cabbage).

Some of the halophyte plants belonging to the Chenopodiaceae family possible to find in Portugal (Franco 1971) are, for example, the species like *Salicornia europaea* L. (littoral, North to South and *Salicornia glauca* Stock. (littoral, centre), *Suaeda maritima* Dum. (littoral, from North to South), *Atriplex glaucum* L. (littoral, from Mondego river to Sado), and *Atriplex hastatum* L. (littoral and Sub littoral, North to South).

Concerning Portugal and regarding the Brassicaceae family (Franco 1971), there can be found *Crambe hispanica* L. *(*littoral, from Douro to Alentejo), *Cakile maritima* Scop. (littoral, North to South), and several other species from the genus *Diplotaxis* (Fig. 1). *S*pecies such as *Diplotaxis virgata* (UTAD 2008), *Diplotaxis viminea* (L.) DC. (Agricultural Research Service 2008), *Diplotaxis catholica* (Lonchamp 2000) and *Diplotaxis vicentina* (ICN 2006). This last one is endemic and suffering rarefaction, occurring only in the Southwest Alentejo and Vicentine Coast Natural Park. Furthermore, commercial varieties (like *Diplotaxis tenuifolia*) are already available in the market and being used mostly in salads in the Portuguese diet. The climate and some soils of Portugal present unique conditions for commercial production of this species, being possible to harvest consecutively for 8/9 months (Dias 1996), but this agricultural activity is still incipient in Portugal, with only some companies exploring *Diplotaxis* (e.g. VendinGomes & Horter Co., that uses hydroponic systems). Most of these rocket producing companies sell their products to England and Holland as 4th generation vegetables (Dias 1996). Furthermore Portuguese coast also presents unique conditions for putative use of large scale production of salt tolerant species (hypothetically, *Diplotaxis* species) developing an emerging market opportunity.

Figure 1 – Several examples of species within *Diplotaxis* genus occurring in Portugal: a) *Diplotaxis catholica* (Uniprot 2008) *;* b) *Diplotaxis tenuifolia* (http://dearkitty.blogsome.com/2007/05/06/flowers-and-birds-of-the-little-desert/); c) *Diplotaxis viminea* (Rignanese 2008).

THE CONCEPT OF SALT-TOLERANCE

When studying salt tolerance it is important to make clear the parameters considered for salt tolerance. The definition of halophyte (halo=salt + phyte=plant) is somehow ambiguous. Following Schimper definition (1891) *in* Koyro et al. (2006), halophytes are plants that must be able to complete their life cycle in a NaCl substrate rich. On the other hand Chapman (1960) states that a halophyte plant is a "salt-tolerant plant". Gorham (1993) considers halophytes as "plants that occur naturally in soils with an electrical conductivity exceeding 4 dSm^{-1}, and thus includes moderate to highly salt-tolerant species".

Yensen (2006) defines halophytes as the ones able to complete their life cycle under high salinity conditions and glycophytes as the ones which "do well on freshwater and have decreasing productivity with increasing salt levels". Although a clear distinction is often made between halophytes and glycophytes, there is in reality a continuous spectrum of tolerance ranging from the most sensitive species, which are severely affected by as little as 1/10th

seawater [50 mM NaCl], to those species able to complete their life cycle in full strength seawater [500 mM NaCl] (Duncan 1974, Gorham et al. 1985).

This way, there isn't a universally accepted concept for halophyte plants. The lack of consensus on the parameters to consider for halophyte definition will hamper comparative studies involving this class of plants, and authors must always clarify the terminology they adopt. In this report the terminology used by Maas (1986) Agricultural Crops Classification was adopted and can be seen in Figure 2.

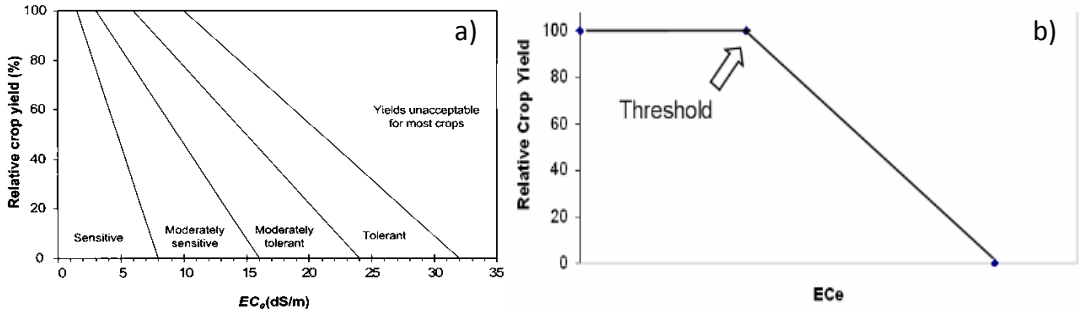

Figure 2 - General relationship between relative crop yield and growth medium electric conductivity. a) Adapted from Maas (1986) Agricultural Crops Classification. b) Adapted from Maas and Hoffman (1977).

Eugene Maas and Glenn Hoffman created the Maas-Hoffman coefficients (Maas and Hoffman 1977) that can be summarized by the graphic in Figure 2a. These scientists have found that research reports on crop growth related to electric conductivity could approximately be characterized by two straight lines (Fig. 2b), which is basically an index reflecting the salt-sensitivity of a given crop.

The flat line indicates the maximum productivity or yield given by the maximum DW value for the edible parts, at all salinities up to a "threshold" number, increasing the amount of salts beyond this threshold causes a linear decrease in crop growth. This way there are two coefficients ("Maas–Hoffmann coefficients") characterizing crop production the threshold value and the slope of the curve at values greater than the threshold value.

MECHANISMS OF SALT TOLERANCE

One way of dealing with the incoming salts is to dilute them by developing cells with very high water content – the so called "succulent" tissues (Munns 2002). Many halophytes employ this technique – the glasswort *Salicornia*, for example, has succulent stem cells, while the mangrove *Rhizophora* has succulent leaves (Little 2000). It was clear to Gorham et al. (1985) that a wide range of physiological, anatomical and biochemical features are involved in the control of solute and water balance and distribution on a whole plant basis, but just as earlier stated by this same author "*Tolerance to salinity is determined by a number of separate, but interrelated mechanisms. Thus the degree of tolerance is determined by the least successful mechanism(s)*" (Gorham 1993).

Intracellular compartmentation as described by Gorham et al. (1985):

- under saline conditions the large quantities of salt absorbed into the leaves are accumulated mainly in the vacuole when tissue concentrations exceed 200 mM;

- the concentration of inorganic ions in the cytoplasm is held in a range of 100 to 200 mM, and the cytoplasm is selective for potassium over sodium, magnesium over calcium, and phosphate over chloride or nitrate;
- under hyperosmotic conditions the maintenance of osmotic balance across the tonoplast requires the accumulation in the cytosol of nontoxic organic solutes, normally referred to as compatible solutes.

Most aquatic halophytes can actually exclude ions from uptake by their roots, keeping salt concentrations in the plants below that of the environment. The barrier to salt is created by the membrane of the root cells, which have a very low permeability to sodium and chloride, and presumably have active transport systems that pump salt outwards (Little 2000).

In salt marshes, where external salt concentrations (and therefore osmotic pressures) are higher than those of the root cells, “normal” plants would wilt. Salt marsh plants overcome the problem by a mechanism in the leaf cells that actively absorb salts instead of excluding them. The decreased osmotic potential caused by the salt helps to draw water into the cells from xylem, in effect increasing the suction pressure and drawing up water from the roots (Little 2000). A complementary mechanism is to rely on young leaves for photosynthetic CO_2 assimilation and discard the older leaves when they become overloaded with salt (Gorham 1993).

Salinity affects almost every physiological and biochemical characteristics recognizable when discussing halophyte salt tolerance and all factors contribute for their survival and establishment in the saline environment (Debez et al. 2006). Saline environments are of difficult survival, for in all of them there is little availability of water and essential nutrients and sometimes direct action from the waves, thus creating an environment of physiological drought (Costa 2001, Debez et al. 2004, Ueda et al. 2003).

No species may have developed the whole set of biologic characteristics associated to salt tolerance but at least some of them are necessary adaptations to plants living in theses unpredictable environments (Debez et al. 2004, Ungar 1991, Yensen 2006). A group of biological characteristics that confers the plant halophytism have apparently evolved independently in diverse groups of vascular plants (Gorham 1993, Ungar 1991, Yensen 2006) instead of evolving from a single ancestor. This hypothesis strengthens the idea that many glycophytes have halophyte genes that are permanently switched off, but under mutagenesis can revert to halophytism (Yensen 2006). Several authors like Cuartero et al. (2006) and Munns et al. (2006) chose the genetic way to try to increase the salt tolerance of crops like tomato and wheat.

Plant responses to salt usually result from the combination of several stresses: osmotic stress, ion toxicity and nutrient imbalances. These stresses acting often in synergy lead to a cascade of effects-responses in the cell involving for example ion and osmotic homeostasis as well as antioxidative responses (Fig. 3).

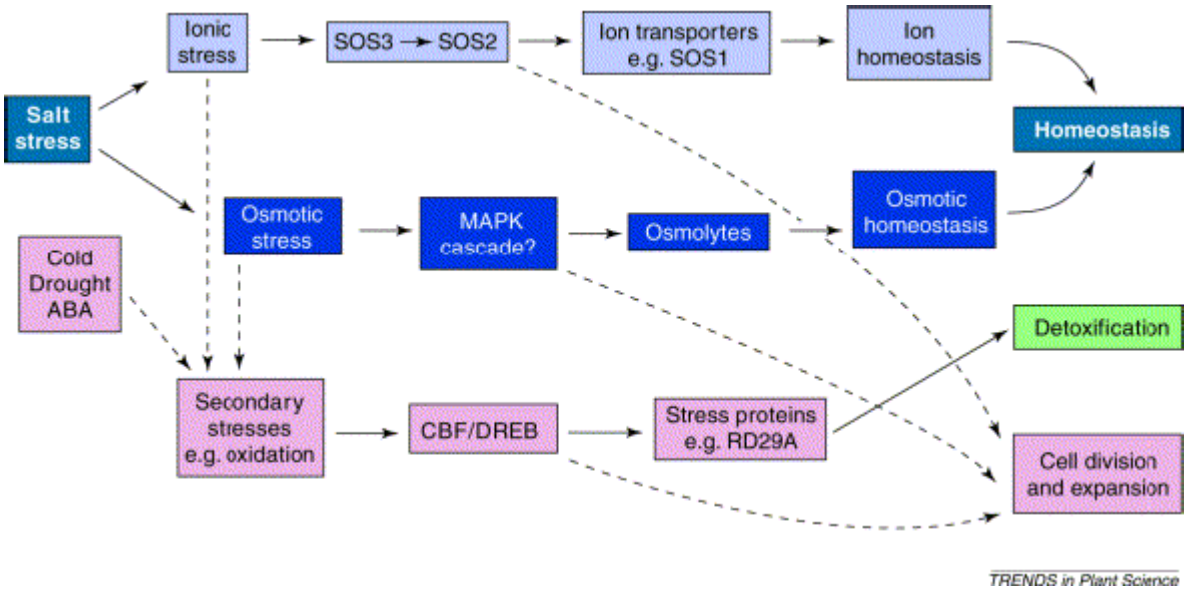

Figure 3 - Three aspects of salt tolerance in plants (Zhu 2001). This diagram shows the osmotic stress and ion imbalances caused by high concentrations of salts in plants, and also the secondary stresses that it causes like oxidation and creation of ROS (reactive oxygen species).

Plant responses to salt stress may ultimately involve plant survival and/or growth (e.g. fresh or dry matter) and yields (e.g. seed production). Often, the expression or level, of the agents involved in antioxidative responses such as the activities of antioxidative stress enzymes (e.g. peroxidase or catalase), or the accumulation of antioxidative metabolites such as glutathione, organic acids or secondary metabolites such as polyphenols may be measured in plants under salt stress, as this stress condition may lead to an increase of oxidative status of the plant cell.

It is generally known that biotic or abiotic stresses may stimulate synthesis or accumulation of polyphenolic compounds (Dixon and Paiva 1995, Naczk and Shahidi 2004). These compounds are ubiquitous in nature playing many different roles in plant biology and human life. They can act for example in the plant biology as defensive compounds from herbivores, contributing to plant colour or taste etc. In the human dietary it has several demonstrated beneficial effects like anti-allergic, anti-inflammatory, cardioprotective etc (Balasundram et al. 2006). Flavonoids are a category within Polyphenolic compounds categorized according to chemical structure (Fig. 4). Due to their potential beneficial effects on human health flavonoids have aroused considerable interest recently, in particular, the flavonoids quercetin (3,3',4',5,7-pentahydroxyflavone) almost ubiquitous in plants and plant food sources (e.g. vegetables, fruit skins and onions), and due to its antioxidant properties and its abundance has called the attention of nutrient and health researchers (Lamson and Brignall 2000). Recently, Heimler et al. (2007) evaluated the contents of several polyphenols in *Lactuca sativa*, *Cicorium intybus*, *Plantago coronopus*, *Eruca sativa* and *Diplotaxis tenuifolia*, reporting that not only quercetin was abundant but also that it presented the highest chelating ability.

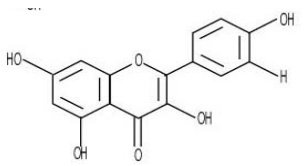

Figure 4 - Quercetin 3,3',4',5,7-pentahydroxyflavone chemical structure.

Furthermore, plants need to overcome osmotic and nutritional imbalances induced by salt stress. It is well documented that salt stress leads to accumulation of toxic ions like Na, and/or to nutrient depletion (e.g. Santos et al. 2001, and Ungar 1991). For example, some elements

are involved in photosynthesis and in protein synthesis regulation and their salt induced-depletion (in particular Mg, a chlorophyll component) may lead to the decrease of chlorophyll synthesis and a quenching of variable fluorescence. Calcium for example, being a second messenger and an important element in cell wall and in membrane structure/stability, its deficiency may lead to serious cell damage. Also, the depletion of the osmoregulator potassium may lead to osmotic unbalances (e.g. (Santos et al. 2001). Maintenance of membrane integrity and the selective uptake of essential minerals as well as ion compartmentation are some of the parameters that were previously related with salt tolerance (Gorham 1993, Salama et al. 1994, Santos et al. 2001). Therefore the evaluation of nutritional status is recognised as necessary and regularly assessed in plants/crops under salt stress (e.g. Santos et al. 2001). This last parameter concerning nutrient contents is particularly important not only to assess plant performance but in crop plants to assess nutritional value of the organs used in human diet, as is the case of *Diplotaxis tenuifolia.*

Objectives

This work generally focuses on two main research questions: *Diplotaxis tenuifolia*' life strategy and its response when grown under saline conditions.

To answer these questions, plants were grown under saline conditions and several parameters were measured/determined:

a) morphological descriptors such as fresh and dry weights (for all parts considered separately); leaf width, length, surface and thickness; root and stem length.

b) physiological descriptors: total polyphenol contents and in particular the flavonoid quercetin levels in the leaves; accumulation of inorganic ions as sodium, potassium, calcium, magnesium and nitrogen.

The overall evaluation of *Diplotaxis tenuifolia* response under salt stress will contribute in the near future to evaluate the possibility of using this species in saline agriculture, and its potential in human dietary.

Materials and Method

Plant material and Growth conditions

Commercial seeds of *Diplotaxis tenuifolia* (Ureeken's Zaden Dordrecht nr. 242100) were evenly sown in black trays filled with manually deflocculated peat soil (Sowing soil nr.1 + SIR, JongKind B.V.). The trays were frequently irrigated with a spray of demineralized water to make the surface wet, and placed in a greenhouse at the Vrije Universiteits' Hortus Botanicus with a day temperature of approximately 20°C and 15°C at night. Plants were grown under a photoperiod of 12h and relative humidity was higher than 60% (light period) and 80% (night). Light intensity was carefully measured, and bulbs of 400 watts were repositioned until achieved an average photon flux density of 300 µmol m^{-2} s^{-1} (PAR) in the growing area. These conditions in the greenhouse were kept constant throughout all the experiment.

Fifteen days after seeds were sown, uniform seedlings with suitable development (two visible cotyledons and two true leaves) were collected soaking the growing tray in tap water to facilitate the peat disaggregation and the seedlings removal. Still with some peat, they were carefully rinsed in a second tray with cold tap water to assure the maximum peat removal from the root zone. Seedlings were then transplanted to 5 litter capacity white trays (Fig. 5a), each one filled up with NS and aerated with two hoses connected to the greenhouses' grid; 20 were assembled. The seedlings were placed in polystyrene plates held by roles of synthetic fibre; these plates had 25 round holes and a size to fit the white trays preventing the entrance of to much light into the rooting area (Fig. 5a, b). Careful was taken with: not letting the synthetic fibre touch the NS and not tightening too much the plant stem.

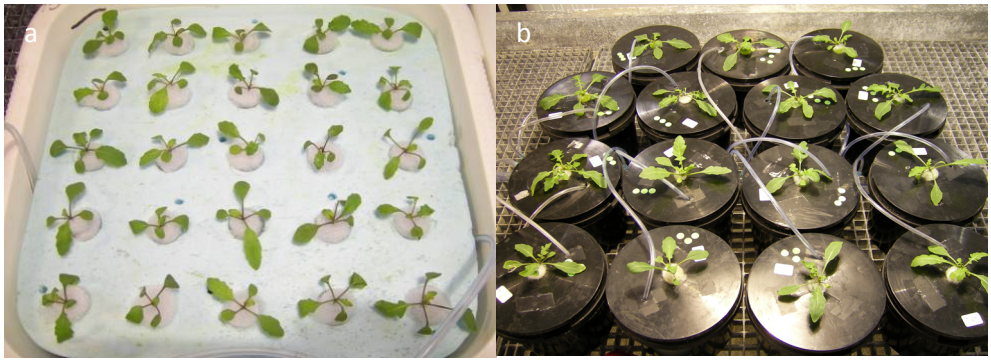

Figure 5 - Experimental design: a) seedlings adjusting to water culture; b) position before randomization of replicates.

After seven days, when plants had developed new roots and adapted to water culture, 100 (again uniform plants) were chosen from the white trays. Considering that each white tray is one replica, plants were chosen from the same range of trays so that there was no difference in the plants origin between salinity treatments.

From these 100 plants, 25 were the initial harvest. The rest 75 plants were placed in individual, lid covered, one litter black polyethylene pots (algae growth prevention) filled up with NS. Five salinity concentrations (see Table 1) with 15 replicas were tested.

Five days after the transference to the black pots, salt adding started in steps of 50mM NaCl (2.92 gl^{-1} NaCl) per day until reached the maximum per treatment, see Table 1. Initially grouped by its salt concentration the replicates were randomly repositioned in the greenhouse after salt adding ended, randomization also happened every time NS had to be changed. Replicates were randomized because the structure of the experiment in a completely randomized design is assumed to be such that the treatments are allocated to the experimental units completely at random, thus producing groups for study that are comparable in unknown as well as known factors likely to influence the outcome, apart from the actual treatment under study.

Table 1 – Salt concentrations tested in milimol (mM), and in gram ($g.l^{-1}$). Conductivities tested in deciSiemens ($dS.m^{-1}$). Schedule in days to achieve each concentration.

NaCl concentrations tested					
mM	0	50	100	200	300
$g.l^{-1}$	0	2.92	5.84	11.69	17.53
Conductivities tested					
$dS.m^{-1}$	0	5.08	10.51	21.04	31.55
Days to achieve	0	1	2	4	6

Plants growing in water culture take up the necessary nutrients from they're growth medium and this one consisted of a Hoagland's NS (Table 2). Also the plants' growth rates determine how often this NS is renewed, which happened three times, throughout growth period of the experiment.

To prepare NS the 60l reservoir would be filled up to 30l with demineralised water. Nutrients were added according to Table 2 and it was also added: 11.7 g of MES ($C_6H_{13}NO_4S$; M= 195.24 $g.mol^{-1}$); 45 ml of [KOH]=2M; 60 ml of [Fe(Na)EDTA]= 7.342 $g.l^{-1}$. Micronutrient solution was made according to Table 2.

Table 2 - Concentrations of individual nutrient solutions and volumes used to prepare the NS.

	Main Element supplied	***(g.l^{-1})***	***Vol. added***
Macronutrients			
KNO_3	N/K	101.10	180
$Ca(NO_3).4H_2O$	Ca	236.16	126
$NH_4H_2PO_4$	P	115.08	60
$MgSO_4.7H_2O$	Mg/S	228.45	30
Micronutrients			60
KCl	Cl	0.075	
$H_3BO_3.H_2O$	B	1.546	
$MnSO_4.4H_2O$	Mn	0.446	
$ZnSO_4.7H_2O$	Zn	0.575	
$CuSO_4.5H_2O$	Cu	0.025	
$(NH_4)_6Mo7O_{24}.4H_2O$	Mo	0.018	

The reservoir would then be very well shaken. The following 30l of demineralised water added. 1000 ml taped and put back on the reservoir, three times; to guarantee equal distribution of nutrients in the reservoirs' content.

One of the requirements for conducting reliable evaluations of plant salt tolerance is to provide stable salinity levels in the growth medium (Miyamoto et al. 1996). The solutions' pH and conductivity suffers changes according the growing rhythm and the period of rapid growth is the one requiring more attention. So, to monitor the solution's pH and the amount of dissolved salts, samples were taken: randomly from three pots per concentration before changing the NS; and also a sample from the fresh NS.

Samples had their conductivity (WTW – Wissenschaftlich Technische Werkstätten LF 91 EGV meter) and pH values (WTW InoLab pH Level 2) measured at a stable room temperature (20°C). Between samples, sensors were cleaned with demineralised water and dried with a tissue.

Calibration of the pH sensor was performed using two standard buffer solutions (two-point calibration) of pH 4 and 7. Because the buffer solutions were from WTW the calibration method was the AutoCal TEC. The measurement mode was AutoRead (drift control), this function checks the stability of the measurement signal that has a considerable effect on the reproducibility of the measured values (WTW 2002).

Growth measurements

Although plants were chosen to be uniform there is always some degree of difference. To understand if the initially smaller/bigger plants remain the smallest/biggest or if there are changes in their growth parameters (Table 3) throughout the growth period, five plants per treatment were chosen to be monitored continuously.

Table 3 - Growth parameters considered in continuous monitoring.

	Length	Width	Thickness
Leaf	X	X	X
Root	X		
Plant total fresh weight			

In the initial harvest, plants were separated into leaves, stems and roots. Parameters measured were the same has for the final harvest (Table 4) that was collected after a growing period of 22 days. Five replicas of each treatment were separated into leaves, root, stem and the rest (Table 4). The leaves were measured individually and separated in "old leaves" (the first two nodes, leafs different in shape and size) and "young leaves" (the following nodes). The rest was the flower stem that had formed at the time and the leaves aggregated to it. Leaf area was measured with a Li-3100 area meter (Li-Corp. Inc., Lincoln Nebraska, USA). Leaf thickness was measured with a Dial pipe gauge (0.01 – 10 mm) Nº 2046-08 Mitutoyo Japan.

Table 4 – Measurements made for final harvest.

		Number	FW (g)	Length (cm)	Width (cm)	Surface (mm)	Thickness (μm)	DW (g)
rest	Leaf		X	X	X	X	X	X
	Root		X	X				X
	Stem		X	X				X
	Leaves	X	X			X		X
	Flower stem			X				X
	rest							X

For both harvests, plants were washed with cold demineralised water and blotted dry before fresh weights were measured and then stored in paper bags.

For the initial harvest plants were oven dried at 70°C until stable weight. For the final harvest samples were dipped in liquid nitrogen just after being collected and freeze dried (LabConco LYPH LOCK 6) until stable weight. When dried, samples were transferred to the exsiccator and weighed in a 4 digit precision balance within the maximum period of two hours. The material was then grounded to a fine powder possible to perform chemical analysis.

Values for RGR and its components were obtained with the "Tool for classical plant growth analysis" from Hunt (2002) and available online at www.aob.oupjournals.org. This table (see Annex 1) derives relative growth rate (RGR) in whole plant dry weight, the main parameter in plant growth analysis, together with its components unit leaf rate (ULR = NAR), specific leaf area (SLA), and leaf weight fraction (LWF). These four are defined and related in the following way:

$$(1/W)(\mathrm{d}W/\mathrm{d}t) = (1/L_\mathrm{A})(\mathrm{d}W/\mathrm{d}t) \times L_\mathrm{A}/L_\mathrm{W} \times L_\mathrm{W}/W$$

$$\mathrm{RGR} \qquad\qquad \mathrm{ULR} \qquad\quad \mathrm{SLA} \qquad \mathrm{LWF}$$

Here t is time, W is total dry weight per plant (g), L_A is total leaf area per plant (cm^2) and L_W is total leaf dry weight per plant (g). The product of SLA and LWF, defined as L_A/W and known as leaf area ratio, is also derived. All five parameters were estimated as mean values solely across one harvest interval (t_1 to t_2), with standard error (s.e.) and 95% confidence limits attached to each estimate.

The value of the exact mean RGR across the harvest interval t_1 to t_2 (day 1 to day 22) is obtained from the following formula:

$$R = (\log_\mathrm{e} W_2 - \log_\mathrm{e} W_1)/(t_2 - t_1)$$

As for mean ULR across the same harvest interval, the approximate value is obtained from:

$$E \approx [(W_2 - W_1)(\log_\mathrm{e} L_{\mathrm{A}_2} - \log_\mathrm{e} L_{\mathrm{A}_1})]/ [(L_{\mathrm{A}_2} - L_{\mathrm{A}_1})(t_2 - t_1)],$$

where the symbol ≈ means "approximately equal to". For the LAR, the quotient at either harvest is defined simply as $f=L_A/W$, with the means of SLA and LWF being obtained by a parallel method of the one for LAR. Since having just two harvests provides no information

about the relation between f and time, the assumed mean value of LAR, and in similar way of SLA and LWF, across the harvest interval t_2-t_1 is:

$$F = \frac{1}{2}(\hat{f}_1 + \hat{f}_2)$$

In this work the time interval comprised 21 days with t_1=1 Initial Harvest, and t_2=22 Final Harvest.

Chemical analysis

Dried material of the young leaves was used for the chemical analysis with exception for the ion analysis that used also the old leaves. This is due to the fact that the old leaves had material for only one analysis.

According to the Folin Ciocalteu method described by (Makkar 2003), 30mg of dry grinded plant material were extracted with a 50% aqueous methanol (MeOH) (v/v) solution; reacted with the Folin Ciocalteu reagent (Merck) and the absorbance measured at 760nm with the spectrophotometer (UV – visible Shimadzu 1601 PC) against demineralized water as a blank. The method used was Autoscan and the system was rinsed with demineralized water between samples. The 11-point calibration curve was obtained with Merck Tannic Acid.

For the separation and quantification of individual polyphenols, 15mg of dry grinded plant material were used and a solution of 100% MeOH containing internal the standard [Flavon]=0.5mg/ml. HCl (20µl, [37%]) and ascorbic acid (5µl, [20 $mg.ml^{-1}$]) were added to the methanol extract and after one hour in a 90°C water bath, the centrifuged supernatant was analyzed in the HPLC Separations Module Alliance 2690 Waters with the Photodiode Array Detector 996 Waters. The method used was the "Condition Column" with the duration of 80 minutes. The flavon peak area was taken at 335nm and the selected flavonoid – quercetin at 254 nm. Trials were performed to detect which flavonoid existed in higher amounts, in this case the quercetin. Quantification of quercetin and correction of lost material during extraction with the internal standard, used an 11-point regression curve with an R^2=0.9982 and R^2=0.9988, respectively.

For nutrient analyses, 30mg of dried plant material (young and old leaves) were wet ached with HNO_3 digestion. The total amount of the alkali sodium and potassium and the earth alkali metals magnesium and calcium analyzed with the Atomic Absorption Spectrophotometer (PERKIN – ELMER 1100B).

The amount of 2 to 3 mg of dry grinded plant material was weighed in a tin capsule. After folding this capsule, it is placed in the autosampler "Carlo Erba NA1500 Rodana Italy". It drops into the reactor where, through a flash combustion, results in a mixture of CO_2, H_2O and NO_x (and some other components such as SO_2). This gaseous mixture flows through a tube where nitric/nitrous oxides are reduced, thus resulting in CO_2, H_2O and N_2. H_2O is trapped with magnesium perchlorate. The gas mixture passes through a chromatographic column (Poropack Q) to separate N_2 from CO_2. These two molecules, N_2 and CO_2, are detected with a Thermal Conductivity Detector (TCD). Differences in thermal conductivity between a reference helium

stream and the sample stream are displayed as visible peaks and recorded as numerically integrated areas.

Statistical analysis

Data were analyzed with the SPSS 16.0 software and the Microsoft Office Excel. Normality was tested with "The One-Sample Kolmogorov-Smirnov Test procedure" with a Lilliefors significance level and the Shapiro-Wilk statistic per dependent variable.

When the assumption of homogeneity with the Levene's test was not fulfilled the Brown-Forsythe and Welch statistics were calculated to test for the equality of group means. These statistics are preferable to the F statistic when the assumption of equal variances does not hold. Analysis of variance of means (One-way ANOVA) was carried out per dependent variable and Post-Hoc Tests used the "Tukey's honestly significant difference" with a significance level computed at $p<0.05$.

Results

Growth analysis

Plants exposed to salt stress had different behaviours with respect to size (e.g. shoots were smaller at 200 and 300 mM), leaf colour (leaves in general presented darker green colour), and morphology (leaves in general were more fleshy than control ones). Also leaf characteristic flavour was less intense (data not shown) and more salty.

The chosen plants for the initial harvest presented a normally distributed biomass and means (Annex 2) of five replicates were not significantly different amongst treatments at $p<0.05$. To ensure that differences among plants did not affect the experiment, 25 plants were randomly collected (from the initial 100 plants) and data confirm no significant differences.

The total above ground biomass production of the final harvest was highest at 100 mM NaCl, but not significantly different from moderate salinities (0 and 50 mM NaCl). Similar results were found for the total leaf dry weight (Fig. 6). Thus, lower salinity treatments (0, 50 and 100 mM NaCl) represented a significantly different homogenous subset ($p<0.05$) from the highest salinity treatment homogenous subset (200 and 300 mM NaCl), for both the biomass production and for the total leaf production (Fig. 6).

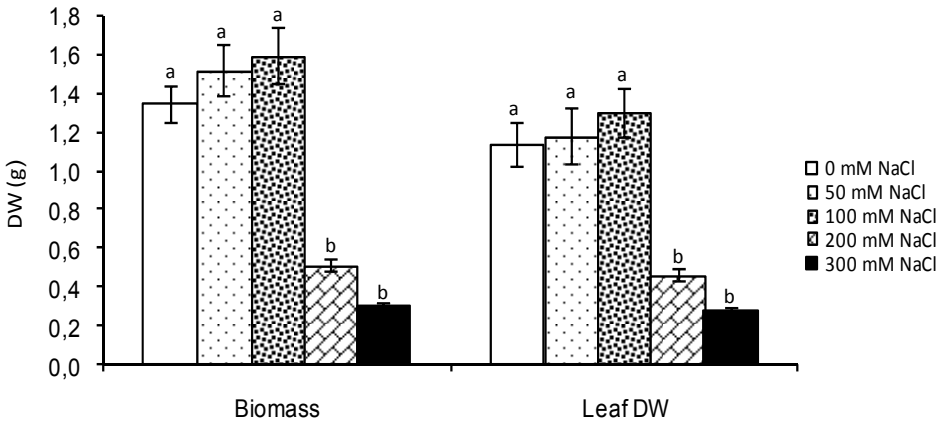

Figure 6 - Effect of salinity level on the Biomass production, as the total above ground DW (g) and the total Leaf DW (g). Same letters within each series indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

The relative effects of five salinity levels on five growth parameters as percentage of control are shown in Table 5. Biomass production for the 200 and 300 mM NaCl had a respective loss from the 100 mM NaCl of 56% and 76%; and from control of 76%and 62% (Table 5).

Table 5 – Relative effect of salinity level on the growth parameters as percentages of control.

NaCl mM	Biomass (%)	Root DW (%)	Leaf Surface (%)	Leaf DW (%)	Thickness (%)
0	100	100	100	100	100
50	113	91	107	104	127
100	119	74	85	115	155
200	38	34	27	41	184
300	22	15	12	24	207

Leaf DW had weight reductions of over 50% against control (Table5). Increasing concentrations of NaCl promoted thickness, being greatest at 300 mM NaCl (207% of control).

Root DW was more sensitive to salinity than stem DW. Root DW decreased with salinity increase. The salinity treatment with 50 mM NaCl was not significantly ($p>0.05$) different from

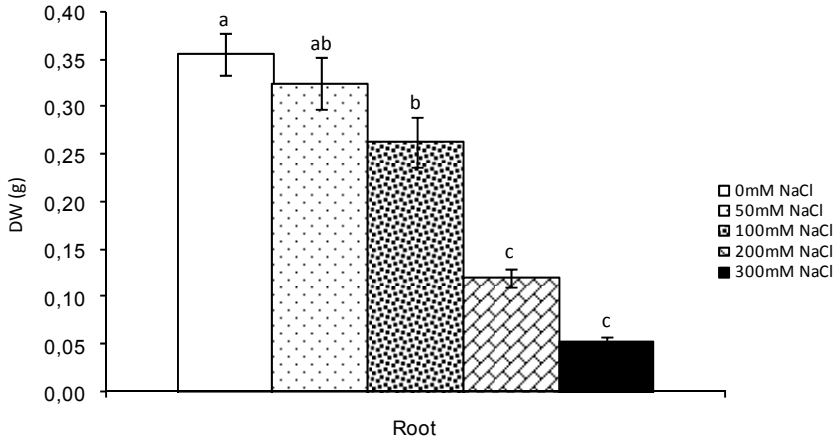

Figure 7 - Effect of salinity level on the Root dry weight (g). Same letters indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

control nor from 100 mM NaCl, being the latest significantly different ($p<0.05$) from control (Fig. 7). The homogenous subset of the highest salinity treatments were significantly different from control; having a decrease in DW to control of 66% and 85% for 200 and 300 mM NaCl respectively.

The total leaf surface (Fig. 8) was highest at 50 mM NaCl, to the next salinity step there was a small but significant decrease in leaf surface. Once more the two highest salinity treatments

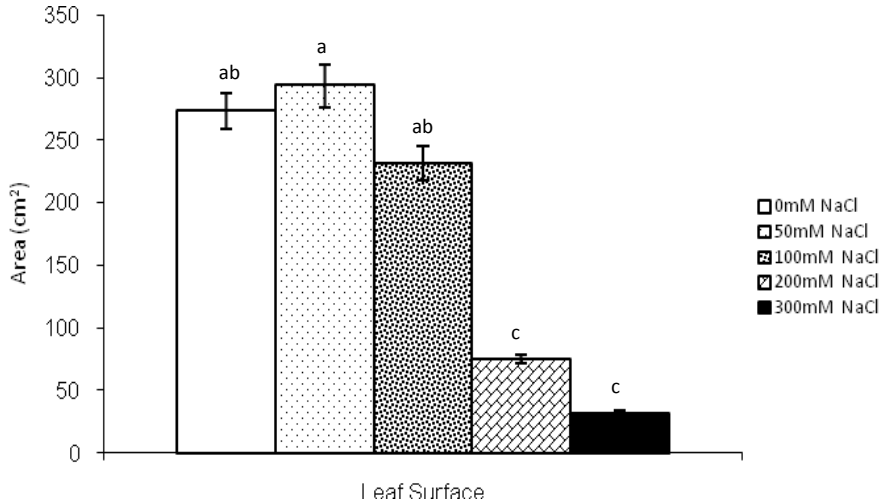

Figure 8 - Effect of salinity level on total Leaf Surface. Same letters indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

form a homogenous subset significantly different from the lower salinities. Leaf surface presented the biggest loss relative to control (88%) for the maximum salinity level.

The stem's dry weight (Annex 2) did not significantly change throughout increasing salinity, although with small variations, it presented the highest value at 50 mM NaCl and the lowest at 300 mM NaCl.

Leaf thickness steadily increased with increasing salinity (Fig. 9) forming no homogenous subsets. Maximum thickness, 207% higher than control, occurred at the highest salinity treatment. At 0 mM NaCl, leaves were 0.04 mm thick suffering an increase to 0.08 mm at 300 mM NaCl.

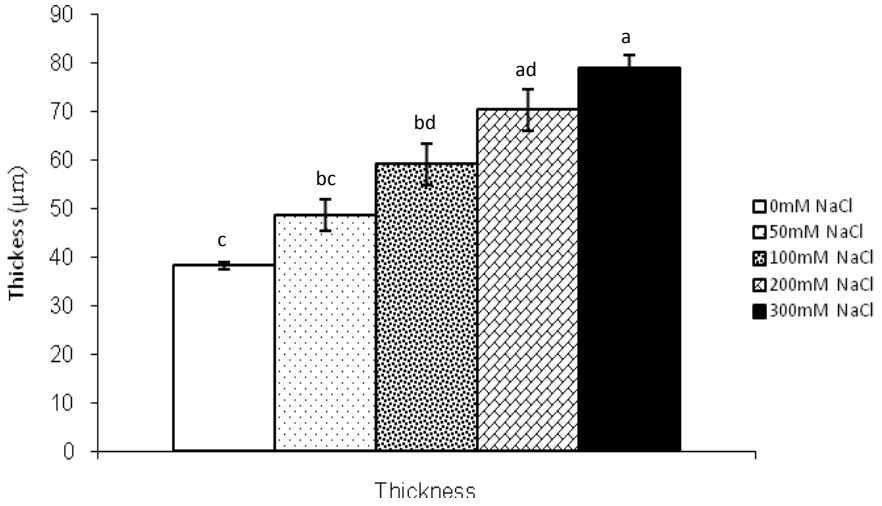

Figure 9 - Effect of salinity level on leaf *Thickness*. Same letters indicate no significant difference (p< 0.05) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

RGR

Relative Growth Rate (RGR, Fig. 10) presented a homogenous subset for the lowest salinity treatments (0, 50 and 100 mM NaCl), occurring practically no variation amongst these three

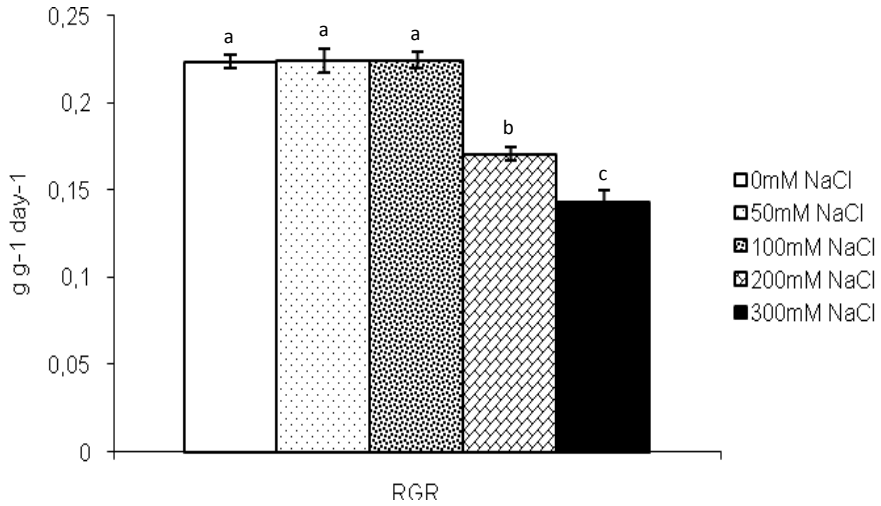

Figure 10 – Effect of salinity level on the RGR – Relative Growth Rate. Same letters indicate no significant difference (p< 0.05) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

first treatments, with an average RGR of 0.22 $g.g^{-1}.day^{-1}$. The higher salinity treatments were significantly different between them and from the lowest salinities homogenous subset.

High concentrations of NaCl caused a decrease on the RGR for low salinities from the mean 0.22 $g\ g^{-1}\ day^{-1}$ to 0.17 $g\ g^{-1}\ day^{-1}$ for 200 mM NaCl and to 0.14 $g\ g^{-1}\ day^{-1}$ for 300 mM NaCl; this represented a loss of 24% and 38% respectively, when compared to control (Table 6).

Table 6 – Effect of salinity level on several growth parameters. Parameter means (n=5) with s.e. were calculated based on harvest at day 1 and 21 after the start of the experiment. Indicated percentages are relative to control.

NaCl mM	RGR[1] $g\ g^{-1}day^{-1}$	%	ULR $cm^{-2}\ day^{-1}$	%	LAR $cm^2\ g^{-1}$	%	LWF[2]	%	SLA $cm^2\ g^{-1}$	%
0	0.221±0.008	100	0.0016±0.0002	100	156±19	100	0.664±0.086	100	238±44	100
50	0.224±0.017	101	0.0016±0.0002	99	159±41	102	0.709±0.133	107	228±54	96
100	0.219±0.010	99	0.0018±0.0003	116	145±22	93	0.706±0.088	106	207±41	87
200	0.169±0.009	76	0.0015±0.0002	92	140±13	89	0.680±0.063	102	203±21	85
300	0.138±0.012	62	0.0014±0.0002	87	132±21	84	0.698±0.088	105	188±29	79

[1]RGR –Relative Growth Rate; ULR –Unit Leaf Rate g; LAR –Leaf Area Ratio; LWF –Leaf Weight Fraction [2](dimensionless); SLA –Specific Leaf Area.

LAR is the product of LWF and SLA. SLA decreased consistently with increasing salinity to a minimum of 21% of control and LWF registered values always higher than control, to a maximum of 7%. In this way, SLA is possibly the responsible parameter for the LAR decrease, except for the 50 mM NaCl when LAR was highest; here SLA was only 4% smaller than control and LWF had its maximum value.

Maximum value for ULR occurred at 100 mM NaCl being 16% higher than control. To be remarked however that the 200 and 300 mM NaCl had small decreases of 8% and 13% (Table 6).

Ions

Sodium accumulation in the old leaves was for all treatments higher per mg of DW than that of the young leaves (data in Annex 2). This difference was smaller in control (100 nmol mg^{-1} DW)

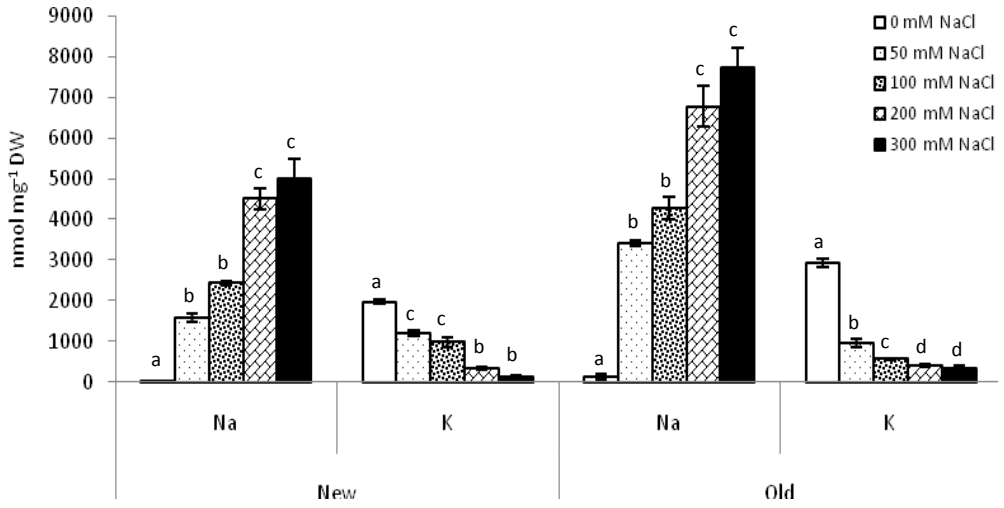

Figure 11 – Effect of salinity level on Na and K (nmol g^{-1} DW) accumulation in the young and old leaves. Same letters indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

but for all other treatments the difference was very high (2000 nmol mg^{-1} DW) increasing with the increase in salinity (Fig. 11).
The larger differences amongst treatments were found for Na^+ especially in the young leaves. At 50 mM NaCl it registered an increase (Table 7) of 5193% and the 300 mM NaCl of 16489%.
Both sets of leaves, young and old, had the same statistical behaviour. Control was significantly different from the homogenous subset of the moderate salinities (50 and 100 mM NaCl) and significantly different from the homogenous subset of the higher salinities (200 and 300 mM NaCl).

Although the amounts of Na^+ in the old leaves were much higher than in the young ones, their relative proportion from control was much smaller (Table 7). It should be noticed that even under control conditions, older leaves accumulated higher levels of Na^+ (128 nmol mg^{-1} DW) than young ones (30 nmol mg^{-1} DW).

Adding NaCl to the NS resulted in a significantly lower K^+ concentration in the leaves (Fig. 11). This loss was more pronounced in the first salinity step for the old leaves with a loss of 67% (Table 7). For both the young and the old leaves a homogenous subset with the 200 and 300 mM NaCl was found and control was also significantly different from all other treatments.

For the young leaves there was a second homogenous subset including the 50 and 100 mM NaCl. In old leaves, significant differences for K^+ accumulation were found for control, 50 and 100 mM NaCl. Amounts of K^+ in the old leaves were generally larger than in the young leaves (Annex 2). At 0 and 300 mM NaCl the old leaves had respectively 1.5 and 2.4 times more K^+ than the young leaves. However this relation changed throughout increasing salinity, with exceptions for the 50 and 100 mM NaCl who presented superior values of K^+ in the young leaves.

Magnesium concentrations in the young and old leaves (Fig. 12) were less sensitive to NaCl levels than any of the other ions. For both sets of leaves, homogenous subsets occurred for the

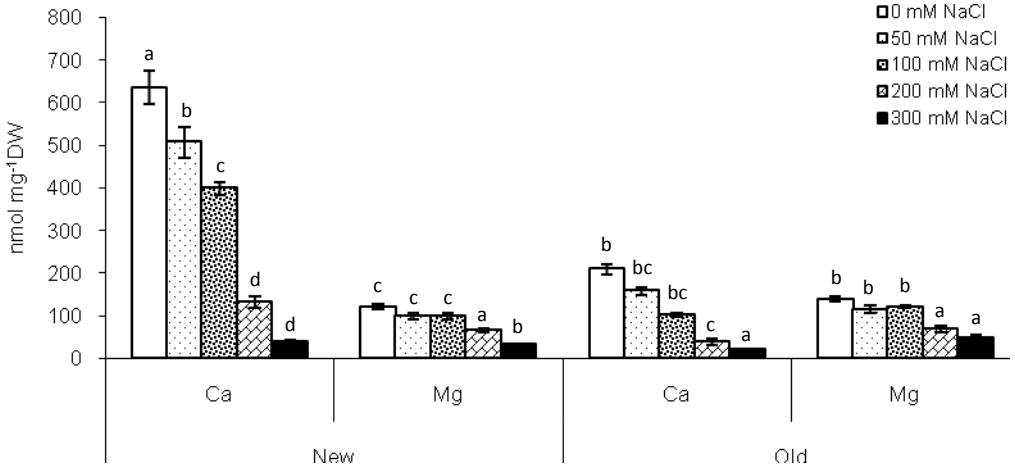

Figure 12 – Effect of salinity level on Ca^{2+} and Mg^{2+} (nmol g^{-1} DW) accumulation in the young and old leaves. Same letters indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

lower salinities (0, 50 and 100 mM NaCl). The 200 and 300 mM NaCl treatments for the young

leaves were significantly different from each other and from the lower salinities. Nonetheless, for the old leaves a second homogenous subset occurred with the highest salinities, significantly different from the lowest. The amounts of Mg^{2+} in the old leaves were always superior to the young leaves though the difference was small.

Table 7 - Relative effect of salinity level on the accumulation of Na^+. K^+. Ca^{2+}. Mg^{2+} and N_2 as percentage of control.

	Na^+		K^+		Ca^{2+}		Mg^{2+}		N_2
mM NaCl	Young	Old	Young	Old	Young	Old	Young	Old	Young
0	100	100	100	100	100	100	100	100	100
50	5193	2667	61	33	80	75	81	83	89
100	7998	3341	50	19	63	49	81	87	82
200	14866	5291	17	13	21	19	54	50	76
300	16489	6039	7	12	6	10	26	35	51

Contents of Ca^{2+} (Fig. 12) presented big differences from the young leaves (higher amounts) to the old leaves (lower amounts). Accumulation of Ca^{2+} in the young leaves was, for control, 3 times bigger than in the old leaves. This is easily seen throughout all salinity treatments being the smallest difference (50%) for the 300 mM NaCl. The differences in the young leaves are even more visible when comparing Ca^{2+} values for control with the highest salinity treatment, being this last one only 6% of control.

Accumulations of Ca^{2+} in the lowest salinity treatments were all significantly different from the homogenous subset of the highest salinities (200 and 300 mM NaCl). In the old leaves, differences are not so visible, the lower salinities group presented no significant difference ($p>0.05$) from the 50 and 100 mM NaCl. The highest salinity was however significantly different ($p<0.05$) from all other treatments.

Excluding the control medium, the ratio between the Na^+ content in the leaves and the Na^+ concentration in the growth medium showed a decreasing trend (Fig. 13).

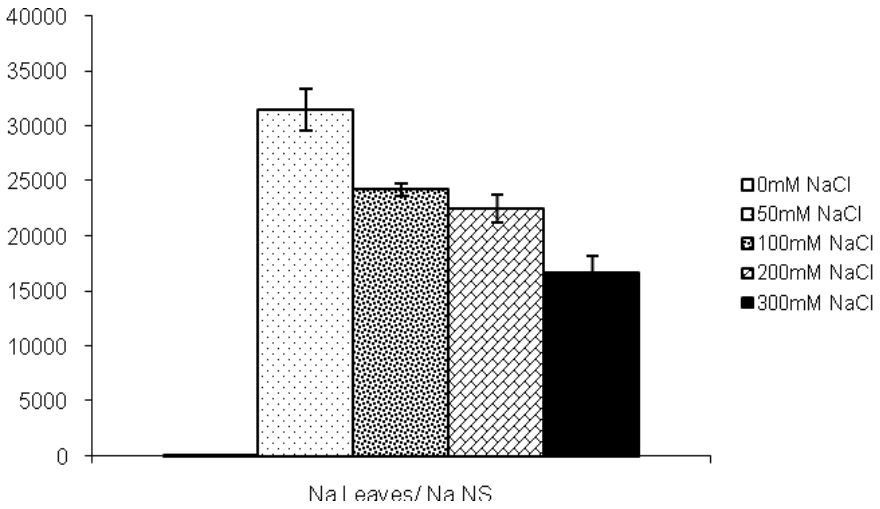

Figure 13 – Ratio between the amount of Na^+ inside the leaves and the existing Na in growth medium. Bars indicate s.e. of means (n=5).

This ratio was not considered for control, although there is sodium in the medium growth from the reagents used to make nutrient solution, in this case NaFe-EDTA. To consider this amount for control it would have to be considered also for the salinity treatments.

The amounts of N_2 in control leaves were 70.98 $mg.g^{-1}$ DW. This amount decreased with the increase of salinity levels (Fig. 14). The steepest decrease happened from the 200 to 300 mM

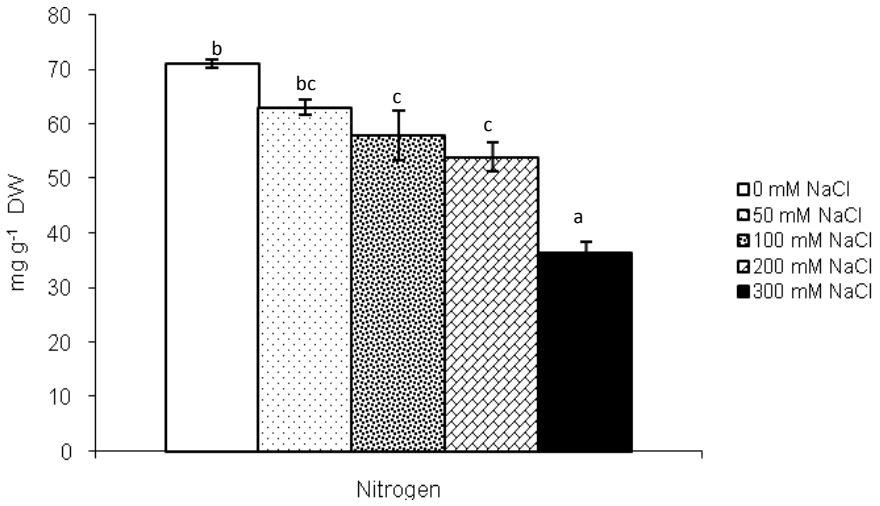

Figure 14 - Effect of salinity level on average leaf Nitrogen. Same letters indicate no significant difference (p< 0.05) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

NaCl (25%), which was similarly to the cumulative decreases from control to 200 mM NaCl.

The Na:K ratio had a very distinct pattern between the two sets of leaves (Fig. 15). The old leaves reached a maximum ratio of 3.9 with the young leaves reaching 36.6. Even though Na:K ratio was always higher for the young leaves at 200 and 300 mM NaCl it presented extremely higher values than the old leaves.

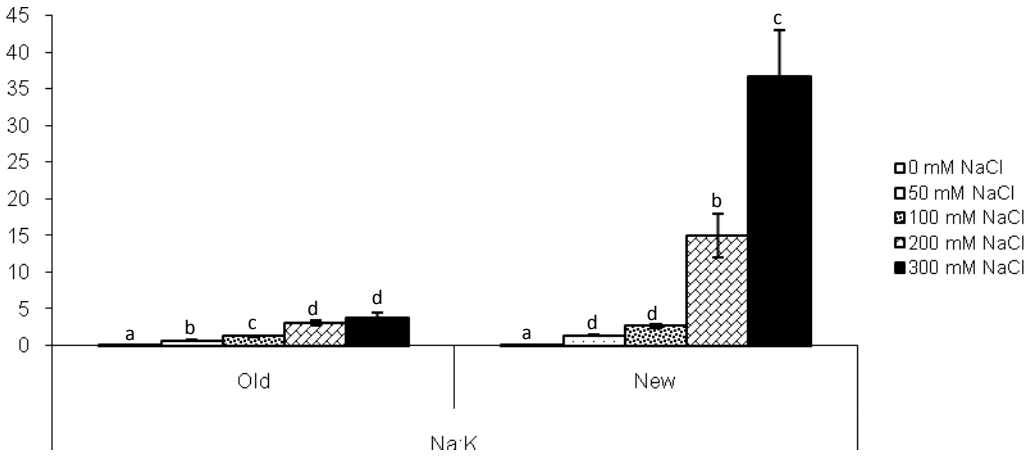

Figure 15 - Effect of salinity level on the Na:K ratio for the Young and Old leaves. Same letters indicate no significant difference (p< 0.05) according to Tuckey's multiple range test. Bars indicate s.e. of means (n=5).

Also, when looking at young leaves, 200 mM NaCl Na:K ratio was 5.7 times higher and significantly different from the one of leaves exposed to 100 mM NaCl. Finally, the ratio Na:K in 300 mM NaCl exposed leaves was 2.5 times higher and significantly different from the 200 mM NaCl. The two highest salinity treatments were not significantly different ($p > 0.05$) for the old leaves.

Chemical Analysis

From the results of the HPLC analysis it was possible to identify and isolate the biggest peak area, corresponding to the flavonoid quercetin. This flavonoid had an average retention time of 41 minutes. In the analysis of quercetin contents two homogenous subsets separating low from high salinities were observed, being the 100 mM NaCl neither significantly different ($p<0.05$) from the low nor from the high salinities.

The total polyphenol content (Fig. 16) also showed a general decrease trend with increasing salt stress. However, within this trend only the highest NaCl concentration (300 mM) led to a significant decreased of polyphenol contents ($p<0.05$) with respect to control.

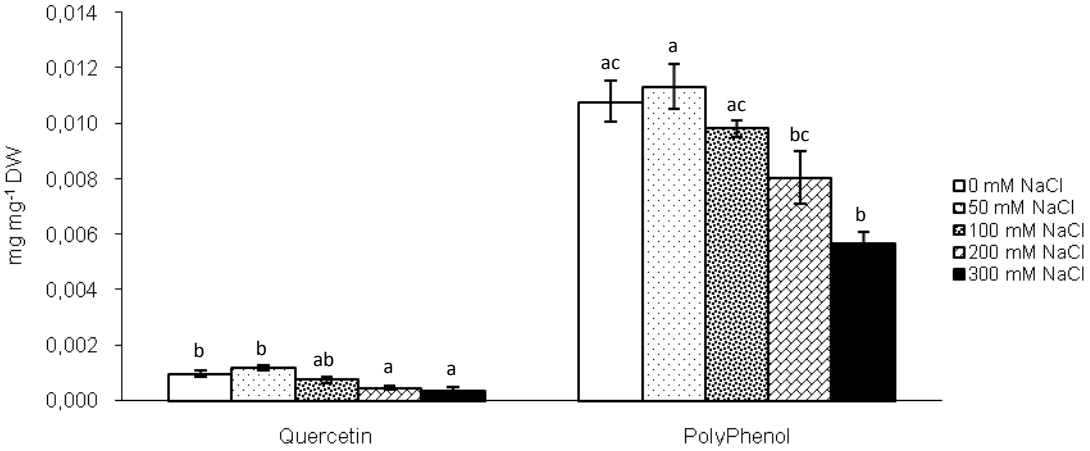

Figure 16 - Effect of salinity level on the total polyphenol content (mg mg^{-1} DW) and on the quercetin content. Same letters indicate no significant difference ($p< 0.05$) according to Tuckey's multiple range test.

Although there were minimal differences in the total polyphenol and quercetin content within the first salinity concentrations, there was a respective increase of 5% and 20% for the 50 mM NaCl; and a following decrease of 9% and 24% for the 100 mM NaCl (Table 8). It should be remarked that the highest salinity had a decrease of 47% in polyphenol content and quercetin of 62% (Table 8).

Table 8 – Effect of salinity level on the total Polyphenol content (mg.mg^{-1}DW) and on the quercetin content with s.e. Indicated percentages are relative to control.

mM NaCl	polyphenol	%	quercetin	%
0	10.79 ± 0.75	100	0.98 ± 0.11	100
50	11.33 ± 0.81	105	1.18 ± 0.11	120
100	9.82 ± 0.31	91	0.75 ± 0.12	76
200	8.03 ± 0.94	74	0.47 ± 0.08	48
300	5.69 ± 0.40	53	0.38 ± 0.11	38

The amounts of quercetin in the leaves had higher increases but also higher decreases, decreasing more rapidly than those of polyphenols (Table 9). Quercetin representation in the total polyphenol content decreased with increasing salinity. At 0 mM NaCl it represented 9% of the total polyphenol content and the minimum 6.2% occurred at 200 mM NaCl, only to suffer a slight rise to 6.9% at 300 mM NaCl (Table 9).

Table 9 – Effect of salinity level on the relative percentage of the flavonoids quercetin, on the total polyphenol content. Indicated percentages (%) are relative to control.

mM NaCl	%
0	9.43
50	8.71
100	7.57
200	6.20
300	6.86

Discussion

The decreasing availability of arable land and fresh water together with the increase in nutrient demand by the flourishing society raise the problem of agronomic sustainable development, here, saline agriculture arises playing an emerging role. This agricultural field may be in the near future of extreme importance in Mediterranean countries due to their geographic and climatic conditions and the increasing soil degradation observed in some regions.

For Portugal, despite the scarce information on soil salinization, the use of saline agriculture may be regarded as highly potential in the future, existing already an ongoing current project to determine the soil' and the irrigation water' salt content.

Among the species with potential for saline agriculture, *D. tenuifolia* is one already being produced in large scale in Mediterranean countries. However the scientific data concerning its performance under salt stress conditions was very limited (e.g. Bianco's (1996) technical report). The scarce information about this species growth under stress conditions, and the characteristics of its growth under natural conditions, led the authors to choose this species as model in this salt stress study.

The response of *D. tenuifolia* to NaCl treatment varied throughout increasing salinity. It showed optimal growth at moderate salinities (50-100 mM NaCl) since improving growth parameters like whole plant DW, total leaf surface and total leaf DW. Also it should be noticed that RGR kept constant until 100 mM NaCl. Similar results were obtained for various salt tolerant and halophyte species of the genus *Sueda* (Liu et al. 2006) and for the halophyte *Cakile maritime* (Debez et al. 2006).

Generally, photosynthetic activity (intimately associated with carbon metabolism and therefore biomass production) is suppressed under salt stress and water stress and it is severely suppressed in salt-sensitive species (Seemann and Critchley 1985).

Also, when Bianco (1996) studied *D. tenuifolia* growing under similar conditions with exception for the growth medium (the author used peat and not hydroponic culture), increasing salinity up to 200 mM NaCl caused a dramatic decrease in the leaf DW of 67%. These results are similar to the decrease found in this study, were 200 mM caused a 62% decrease of leaf DW.

According to McKersie and Leshem (1994) plant responses to salinity include reduction of leaf growth rate and associated reduction in leaf area available for photosynthesis. Considering these assumptions, in the present study, photosynthetic suppression is likely to have occurred above 100 mM NaCl in which biomass production was significantly inhibited. Still the plant was able to survive, grow and reproduce; even being those salinities close to 3/5 of seawater NaCl concentration. As an example of comparison been *D. tenuifolia* and the non-halophyte conventional crop pomegranate, this last one was only able to increase growth up to 40 mM NaCl, decreasing thereafter (Naeini et al. 2006).

In accordance this work' results show that leaves largely accounted for the plants response pattern, since their dry weight and area did not significantly differ from control at optimal salinities (50–100 mM NaCl) just like biomass production and RGR. Leaf surface presented no significant differences at moderate salinities but demonstrated a significant and abrupt surface break again above 100 mM NaCl being the breakdown of 88% from control.

Leaves steadily thickened with increasing salinity reaching a maximum 207% thicker than control, this increment was 1.5 times higher than the one occurred for the halophyte *Beta vulgaris* subsp. *maritima* (Niazi 2007) in similar conditions. These results are in conformity with those obtained for *Phaseolus vulgaris* exposed to moderate salinity levels. Wignarajah et al. (1975) and also for tomato plants submitted to various increasing salinities (Gad 2005). As first stated by Hayward (1941) *in* Longstreth and Nobel (1979), and sustained along the years by various authors (Strogonov 1964 *in* Gad 2005, Longstreth and Nobel 1979), the exposure of roots to high concentrations of NaCl induces increments in leaf thickness. These same authors also concluded that the increment in leaf thickness is a direct result of the increase of the spongy parenchyma layer, namely by the very well known increase in succulence (Munns 2002), among other factors.

Other studies for example Oliveira et al. (2008), Santos and Caldeira (1999), and Santos et al. (2001) also report for other crops (e.g. grapevine, sunflower) a decrease in leaf growth (area and/or weight), decreases in photosynthetic parameters and overall plant growth.

Furthermore, changes in leaf growth (e.g. leaf weight and/or area) influence not only total photosynthesis ability and carbon metabolism performed by the plant, but also plant growth descriptors such as SLA and LWF (see Materials and Method), which supports the importance of assessing leaf growth parameters during salt stress experiments.

Concerning root growth, this parameter had a small but significant decrease at moderate salinities and showed no tolerance to salinity above 100 mM NaCl, decreasing 85% from control. Eker et al. (2006) concluded, for maize grown under salt stress conditions, that measurement of shoot growth is more reliable than root growth for assessing salt tolerance. Also, in other experiments, roots were found to be less sensitive to salinity variations than shoots (e.g. Liu et al. 2006, and Nedjimi et al. 2006) supporting the idea that roots and leaves are differently affected by salt stress. Taking in consideration that the form and function of plant organs undergo considerable changes during plant growth Ashraf and Harris (2004) state that NaCl induced effects in each plant organ are dependent on the genes expressed at the stage of development when stress is imposed. Several other factors play important roles in determining each plant species salinity tolerance like for example the species/genotype used, growth conditions and life cycle status.

Adding NaCl to the NS resulted in no significant effect on the RGR defined as the increase in biomass per unit of biomass at moderate salinities. *D. tenuifolia* had higher RGR values for 0 and 200 mM NaCl than those of the halophyte *Beta vulgaris subsp. maritima* (Niazi 2007).

As discussed above, several parameters of relative growth are conditions by partial organ growth (such as the leaf). Specific leaf area, defined as leaf area per unit leaf mass, was

reduced with increasing salinity. According to Poorter and Garnier (1999) SLA is after LAR, the most important component of RGR. Its inverse 1/SLA is the product of leaf density with leaf thickness, this last one significantly increased with increasing salinity. It is known that the anatomical component thickness increases linearly with the inverse of SLA. Just as occurred with the halophyte *Beta vulgaris* (Niazi 2007), and in this case with *D. tenuifolia* a decrease in SLA was associated with a significant thickness increase. Finally, although SLA is often assumed to be constant (Marcelis et al. 1998) it has been demonstrated also in this work that that's not a common situation.

One way to assess a plant relative tolerance to salinity is to quantify plants growth response to increasing salinities. Based on biomass production of the marketable parts, the leaves, and on the agricultural classification defined by Maas (1986) data from this study show that *D. tenuifolia* may be considered a salt-tolerant species with a threshold value of 100 mM NaCl. As stated above, the threshold salinity value is the point at which the above ground parts yield starts to decline (Maas 1986).

Concerning the effects of salt stress on the nutrient balances of *D. tenuifolia*, salinity restricted the plant nutrient uptake, leading to significant decreases of leaf K with the same tendency observed for Ca^{2+} and Mg^{2+}. Similar results were obtained for *Atriplex canescens* (Richardson and McKell 1980), *Salvadora persica* (Maggio et al. 2000), *Cakile maritima* (Debez et al. 2006), *Limonium bicolor* and *Sueda salsa* (Liu et al. 2006), and also for the crops maize (Eker et al. 2006) and *Beta vulgaris* (Niazi 2007).

Higher accumulations of Na^+ in the leaves, a characteristic of halophytes (Munns and Tester 2008), significantly increased with increasing salinity. This is interpreted as a salt tolerance mechanism by Shannon and Grieve (1999), Daoud et al. (2001) and Liu et al. (2006). This mechanism facilitates osmotic adjustment as in general halophyte species have the ability of effectively accumulate excessive ions in the vacuoles leading therefore to osmotic adjustment with no/low cytosolic toxicity. However, in non-adapted genotypes, under extreme salt conditions, or under prolonged exposures to salt, the accumulation of salt in leaves can lead to ion toxicity, due to undesirable accumulation of these ions in the cell wall or cytoplasm. Also, due to transpiration, Na^+ accumulation in leaves (mostly of non adapted species) was reported to largely exceed the levels of this ion in the external medium (Ungar 1991). These toxic effects are first and predominantly observed in the old leaves (Daoud et al. 2001, Koyro and Huchzermeyer 1999) as they often accumulate higher levels of sodium than young leaves.

According to literature, in *D. tenuifolia*, Na^+ accumulated in much higher amounts in the older leaves than in the young ones, supporting the observed premature induced wilting observed in the older leaves, compared to old leaves of control plants. The higher accumulation of salt in *D. tenuifolia* salt stressed leaves was high enough to be detected by taste, assuming therefore, an important role for commercial purposes.

Besides compartmentation strategies, and to avoid Na^{2+} from reaching cellular toxic levels, *D. tenuifolia* also showed a necessary limitation (Gorham 1993) of the influx of salt into the plant, and in particular into the leaves. This is supported by the fact that, although the leaf levels of Na^+ increased with increasing levels of external sodium, the ratio between the Na^+ in the

leaves and the Na^+ in the growth medium (Fig. 13) decreased. Thus, this species does not take all the Na^+ that it could from the growth medium. Therefore these data strongly suggest that in *D. tenuifolia* some mechanisms are acting in preventing sodium toxicity by selective compartmentation/allocation.

Table 10 – Comparison between ionic concentrations in young and old leaves. (-) lower; (+) higher.

	Young	*Old*
Na^+	-	+
Ca^{2+}	+	-
K^+	+	-

Although the root Na^+ content was not measured in *D. tenuifolia*, the effect of NaCl exposure and Na^+ accumulation in roots growth is often reported as less drastic compared to shoots. For example Eker et al. (2006), Liu et al. (2006) and Naeini et al. (2006) demonstrated that the NaCl application on maize, *S. salsa* and *L. bicolor*, and pomegranate induced less pronounced reductions of root DW in the roots than in the shoots. This may be, according to Eker et al. (2006), an indicator that it's not only the root Na^+ uptake that takes part in the NaCl plant tolerance but also important is the Na^+ transport to the shoots. This is corroborated in a review by (Munns et al. 2006). Considering that the shoots are rapid growing tissues and the ability to reduce Na^+ transport to these structures will indirectly determine Na^+ accumulation, thus protecting the metabolic processes from the toxic effects of Na^+.

Lynch Laüchly (1985) reported that Ca^{2+} increases salt tolerance of plants. This ameliorating effect of Ca^{2+} is in accordance with its functions in membrane integrity and control of selectivity in ion uptake and transport (Daoud et al. 2001). However, just as the occurred in this work, the increase of external salinity generally reduces the amount of Ca^{2+} in the plant (Koyro and Huchzermeyer 1999, Lynch and Laüchly 1985, Marschner 1995). It should however be noticed that the amounts of Ca^{2+} in the young leaves were largely higher than in the old leaves, suggesting that this species expressed mechanisms of retaining necessary Ca^{2+} levels even at high salinity. Retention of Ca^{2+} in young/growing organs (as leaves, flowers or fruits) under stressing conditions (e.g. salt stress) is well described and associated with defensive strategies of the growing regions, once Ca^{2+} is crucial for plant cell wall formation, membrane integrity and ion transport as well as signal transduction and hormone signalling (Taiz and Zeiger 2006).

Concerning N_2 accumulation in *D. tenuifolia,* Bianco (1996) stated that this species tends to accumulate high levels of nitrates in the leaves. In this salt stress experiment, *D. tenuifolia* suffered a decrease of this element in young leaves, with increasing external levels of salt. This is in agreement with the results obtained on the closely related genus *Eruca*, species *Eruca vesicaria* (Ventrella et al. 1993). Nitrogen is present in plants mostly as nitrate or organic nitrogen (e.g. aminoacids, proteins). It is well known that chloride may compete with nitrate and the addition of nitrate to the culture medium was reported to decrease chloride entrance (e.g. Santos 1998). The reduced levels of nitrogen in salt stressed plants may therefore be justified by a competition effect of chloride *vs*. nitrate. However this effect should be better studied, as salt stress often induced protein degradation (e.g. rubisco) in older leaves and

nitrogen mobilisation to the growing parts (young leaves) as, mostly, aspargine and glutamine (Santos 1998).

The obtained values of Na:K ratio in *D. tenuifolia* control plants fit within the range of 0.01 and 0.05 proposed by Taiz and Zeiger (2006) for plants growing under non-saline conditions. Moreover, despite the fact that Na is a cheap osmoticum for halophytes, an excess of this ion over K^+ can inhibit several metabolic processes (Debez et al. 2006). The significant increase in this ratio was coincident with the significant decrease in RGR and biomass production, and has been obtained by several authors in similar studies (Epstein 1966, Naeini et al. 2006). This ratio has been reported to be a reliable physiological index for salt tolerance (Eker et al. 2006, Lessani and Marschner 1978, Marschner 1995).

In what comes to nutritional value (Ca^{2+}, K^+, Mg^{2+}) this salad product has slightly lowered its quality at moderate salinities and drastically at higher salinities. This aspect must be taken in consideration when it comes to production and commercialization of this species. Nonetheless Na content increased dramatically with increasing salinity, which may be used for marketing avoiding salt adding.

In accordance with previous findings (Bennett et al. 2006, Heimler et al. 2007) quercetin was identified just as expected as the most abundant flavonoid in *D. tenuifolia* for all salinities tested showing a slight increase at 50 mM NaCl. Heimler et al. (2007) as also shown that quercetin had the highest chelating ability of 57% in contrast with the 12% for Ascorbic Acid.

There was a 5% increase in the total polyphenol content for the 50 mM NaCl that was accompanied by a 13% increase in biomass production. However the total polyphenol content decreased for all other higher salinities. Still the only significant decrease from control, of 53%, was that of the 300 mM NaCl treatment. Ksouri et al. (2007) showed similar results, when it's more salt sensitive population of the halophyte *Cakile maritima*, steadily but not significantly decreased total polyphenol content with increasing salinity.

In this work biomass production had its significant decreases at the highest salinities of 200 and 300 mM NaCl. Therefore results obtained here for moderate NaCl concentrations corroborate those of Ksouri et al. (2007) hypothesis that the capacity to accumulate polyphenols participates in the plant's salt tolerance mechanism. However for higher concentrations polyphenol content decreases, suggesting that other antioxidants may be involved or that the plant has reached its limit of combating the oxidative stress induced by the high salt concentrations. Leaf quality as source of naturally occurring antioxidants (namely polyphenolic compounds) as ameliorated for 50 mM decreasing thereafter.

Conclusion

To maintain a prosperous agriculture and the of use saline waters for irrigation, the salt tolerance of cultures must be known. This work shows for the first time the salinity tolerance of *D. tenuifolia* in a gradient of increasing salinities under greenhouse controlled conditions. One of the conclusions of this work is that *D. tenuifolia* is a salt tolerant species according to Maas (1986) agricultural crops classification. These results on its own do not explain the mechanism of salt tolerance used by this plant, which anyway was not the purpose of this work; nonetheless they do give an initial overview of the plants' response when grown under a certain degree of salt stress. In a following step, studies can be performed on many other possible parameters for assessing salinity tolerance and maybe understand the distinctive indicators of salt tolerance at whole plant, tissue or cellular level for this economically important species.

Diplotaxis spp., included in the Portuguese spontaneous and sub-spontaneous flora is amongst the country's natural vegetation. In the same way that *D. tenuifolia* is a relevant species in agriculture and medicine nowadays*, D. vicentina* Rothm*,* as a member of the *Diplotaxis* genus, may eventually be a potential crop. According to the Portuguese Ministry of Agriculture (Min.Agric. 1996) *Diplotaxis* spp. has immediate agricultural interest and can be included in the gene-pool for breeding of cultivated plants. Just like *D. tenuifolia*, *D. vicentina* also occurs on the shore line, with some degree of exposure to salt spray. Therefore, to perform a similar study on *D. vicentina* could reveal itself interesting and useful. It is the authors' conviction that the search for endemic vegetables, to be used as food, offers wide chances to add value to local products.

Within the scope of this governmental strategy, and within the perspective of using saline agriculture in Portugal, we tested the potential of this species under salt stress and demonstrated for the first time that:

a) *D. tenuifolia* is a tolerant species bearing salinity concentrations up to 100 mM NaCl or higher (although with a biomass production decrease), suggesting that it may be used in saline agriculture;

b) Maximum growth (leaf biomass, a commercial parameter) occurs at 100 mM NaCl;

c) Although leaf visual aspect and thickness are slightly affected there is still a great commercial possibility for acceptance, requiring however specific market studies;

d) Leaf's nutritional quality, when grown in saline environment is affected when compared to control. This should be taken into account when implementing this crop;

e) Leaf quality as a source of naturally occurring antioxidants (namely quercetin) improved at 50 mM NaCl decreasing thereafter.

This study significantly contributes to the available knowledge on Diploatxis tenuifolia's salt tolerance and it is a valuable asset to future crop implementation studies of this species. It contributes not only to the knowledge of this species under saline conditions but also to the existing databases on potential species for saline agriculture.

References

Agricultural Research Service , N. G. R. P., United States Department Agriculture (2008). Germplasm Resources Information Network - (GRIN) [Online Database], National Germplasm Resources Laboratory, Beltsville, Maryland.

Asano, N., A. Kato, K. Matsui, A. A. Watson, R. J. Nash, R. J. Molyneux, L. Hackett, J. Topping and B. Winchester (1997). "The effects of calystegines isolated from edible fruits and vegetables on mammalian liver glycosidases." Glycobiology **7**(8): 1085-1088.

Ashraf, M. and P. J. C. Harris (2004). "Potential biochemical indicators of salinity tolerance in plants." Plant Science **166**(1): 3-16.

Balasundram, N., K. Sundram and S. Samman (2006). "Phenolic Compounds in Plants and Agri-Industrial by-products: antioxidant activity, occurrence and potential uses." Food chemistry **99**: 191-203.

Barnes, R. F. and J. E. Baylor (1995). Forages in a changing world. Forages: An Introduction to Grassland Agriculture. R. F. Barnes, D. A. Miller and C. J. Nelson. Ames, Iowa State University Press**:** 3–13.

Bennett, R. N., E. A. S. Rosa, F. A. Mellon and P. A. Kroon (2006). "Ontogenic Profiling of Glucosinolates, Flavonoids, and Other Secondary Metabolites in *Eruca sativa* (Salad Rocket), *Diplotaxis erucoides* (Wall Rocket), *Diplotaxis tenuifolia* (Wild Rocket), and *Bunias orientalis* (Turkish Rocket)." J. Agric. Food Chem. **54**(11): 4005-4015.

Bernstein, L. (1975). "Effects of Salinity and Sodicity on Plant Growth." Annual Review of Phytopathology **13** 295-312.

Bianco, V. V. (1996). Rocket, an ancient underutilized vegetable crop and its potential. Rocket Genetic Resources Network. P. S. Lisbon, Portugal, International Plant Genetic Resources Institute, Rome, Italy.**:** 38-60.

Böer, B. (2006). Halophyte research and development: What needs to be done next? Tasks for Vegetation Science – Ecophysiology of High Salinity Tolerant Plants. M. A. Khan and D. J. Weber. Netherlands, Springer Netherlands. **40:** 397-399.

Boyko, H. (1966). Salinity and Aridity: new approaches to old problems. The Hague, Dr. W. Junk Publishers.

Callaway, J. C. and J. B. Zedler (2004). "Restoration of urban salt marshes: Lessons from southern California." Urban Ecosystems(7): 107-124.

Chapman, V. J. (1960). Salt marshes and salt deserts of the world. New York, New York:Interscience Publishers.

Choukr-Allah, R. (1993). Introduction Advanced Course on Halophyte Utilization in Agriculture, Morroco.

Costa, J. C. (2001). Tipos de Vegetaçao e Adaptações das Plantas de Portugal Continental Homenagem (in honorio) Professor Doutor Soares de Carvalho. M. E. Albergaria Moreira, A. Casal Moura, H.M. Granja & F. Noronha. Braga, Universidade do Minho**:** 283-299.

Cuartero, J., M. C. Bolarín, M. J. Assíns and V. Moreno (2006). "Increasing salt tolerance in the tomato." Journal of Experimental Botany **57**(5): 1045-1058.

D'Antuono, L. F., S. Elementi and R. Neri (2008). "Glucosinolates in Diplotaxis and Eruca leaves: Diversity, taxonomic relations and applied aspects." Phytochemistry **69**(1): 187-199.

Daoud, S., M. C. Harrouni and R. Bengueddour (2001). Biomass production and ion composition of some halophytes irrigated with different seawater dilutions. . First International Conference on Saltwater Intrusion and Coastal Aquifers - Monitoring, Modeling and Management. . Essaouira, Morocco.

Debez, A., K. Ben Hamed, C. Grignon and C. Abdelly (2004). "Salinity effects on germination, growth, and seed production of the halophyte Cakile maritima." Plant and Soil **262**(1): 179-189.

Debez, A., W. Taamalli, D. Saadaoui, Z. Ouerghi, M. Zarrouk, B. Huchzermeyer and C. Abdelly (2006). Salt effect on growth, photosynthesis, seed yield and oil composition of the potential crop halophyte Cakile maritima. Biosaline Agriculture and Salinity Tolerance in Plants**:** 55-63.

Dias, J. C. S. (1996). Rocket in Portugal: Botany cultivation uses and potential. Rocket: a Mediterranean crop for the world. B. Padulosi and D. Pignone. Legnaro (Padova) Italy**:** 81-85.

Dixon, R. A. and N. Paiva (1995). "Stress-induced phenylpropanoid metabolism." Plant Cell(7): 1085-1097.

Duncan, W. H. (1974). Vascular Halophytes of the Atlantic and Gulf Coasts of North America North of Mexico. Ecology of Halophytes, New York, U.S.A, Academic Press.

Eker, S., G. Cömertpay, Ö. Konuskan, A. C. Ülger, L. Öztürk and I. Çakmak (2006). "Effect of Salinity Stress on Dry Matter Production and Ion Accumulation in Hybrid Maize Varieties." Turkish Journal of Agriculture & Forestry **30**(6): 365-373.

Epstein, E. (1966). "Dual pattern of ion absorption by plant cells and by plants." Nature **212**: 1324-1327.

Epstein, E., J. D. Norlyn, D. W. Rush, R. W. Kingsbury, D. B. Kelley, G. A. Cunningham and A. F. Wrona (1980). "Saline Culture of Crops: A Genetic Approach." Science **210**(4468): 399-404.

FAO. (2008). "Land and Plant Nutrition Management Service." from http://www.fao.org/ag/AGL/public.stm

Flowers, T. J. (2004). "Improving crop salt tolerance." Journal of Experimental Botany **55**: 307±319.

Franco, J. A. (1971). Nova Flora de Portugal (Continente e Açores). Lisboa, Sociedade Astoria Ltd

Gad, N. (2005). "Interactive effect of salinity and cobalt on tomato plants. II - Some Physiological Parameters As Affected By Cobalt And Salinity." Research Journal of Agriculture and Biological Sciences **1**(3): 270-276.

Galvani, A. (2007). "The challenge of the food sufficiency through salt tolerant crops." Reviews in Environmental Science and Biotechnology **6**(1): 3-16.

Glenn, E., N. Hicks, J. Riley and S. Swingle (1993). Seawater Irrigation of Halophytes for Animal Feed. Advanced Course on Halophyte Utilization in Agriculture, Agadir, Morocco.

Glenn, E., N. Hicks, J. Riley and S. Swingle (1995). Seawater irrigation of halphytes for animal feed. Halophytes and Biosaline Agriculture, New York: Marcel Dekker.

Glenn, E., S. Miyamoto, D. Moore, J. J. Brown, T. L. Thompson and P. Brown (1997). "Water requirements for cultivating Salicornia bigelovii Torr. with seawater on sand in a coastal desert environment." Journal of Arid Environments(36): 711-730.

Gorham, J. (1993). Mechanisms of Salt Tolerance of Halophytes. Advanced Course on Halophyte Utilization in Agriculture, Agadir, Morocco.

Gorham, J., R. G. Wyn and J. E. Mcdonnell (1985). Biosalinity in Action: Bioproduction with Saline Water

Grid/Arendal. (2008). "Total global saltwater and fresh water estimates." from http://maps.grida.no/go/graphic/total_global_saltwater_and_freshwater_estimates.

Grieve, C. M. and D. L. Suarez (1997). "Purslane (Portulaca oleracea L.): A halophytic crop for drainage water reuse systems." Plant and Soil(192): 277–283.

Hayward, H. B. (1941). "Anatomical and physiological responses of the tomato to varying concentrations of sodium chloride, sodium sulphate and nutrient solutions." Botanical Gazette (102): 437-462.

Heimler, D., L. Isolani, P. Vignolini, S. Tombelli and A. Romani (2007). Polyphenol Content and Antioxidative Activity in Some Species of Freshly Consumed Salads. **55:** 1724-1729.

Hodges, C. N., T. L. Thompson, J. J. Riley and E. P. Glenn (1993). "Reversing the Flow: Water and Nutrients from the Sea to the Land." AMBIO A journal of the human environment **22**(7): 483-490.

Hunt, R., D. R. Causton, B. Shipley and A. P. Askew (2002). "A Modern Tool for Classical Plant Growth Analysis." Annals of Botany **90**(4): 485-488.

ICN, I. d. C. d. N. (2006). Flora. Plano Sectorial da Rede Natura.

Khan, M. A. and N. C. Duke (2001). "Halophytes – A resource for the future." Wetlands Ecology and Management **9**(6): 455-456.

Koyro, H. W., N. Geissler, S. Hussin and B. Huchzermeyer (2006). Mechanisms of cash crop halophytes to maintain yields and reclaim saline soils in arid areas. Ecophysiology of high salinity tolerant plants. M. A. Kahn and D. J. Weber, Springer Netherlands. **40:** 345-366.

Koyro, H. W. and B. Huchzermeyer (1999). Influence of high NaCl - Salinity on growth water and osmotic relations of the halophyte Beta vulgaris ss. Maritima - Development of a quick check.

Ksouri, R., W. Megdice, A. Debbez, H. Falleh, C. Grignon and C. Abdelly (2007). "Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritima*." Plant Physiology and Biochemistry(45): 244-249.

Lamson, D. W. and M. S. Brignall (2000). "Antioxidants and Cancer III: Quercetin." Alternative Medicine Review **5**(3): 196-208.

Lessani, H. and H. Marschner (1978). "Relation between salt tolerance and long distance transport of sodium and chloride in various crop species." Australian Journal of Plant Physiology **5**: 27-37.

Lieth, H., C. Zeilinger and J. Papenbrock (2002). Developing Cash Crops from Halophytes. Prospects for Saline Agriculture. R. Ahmad and K. A. Malik. Netherlands, Kluwer Academic Publishers**:** 309-319.

Little, C. (2000). The biology of soft shores and estuaries. New York, Oxford University Press Inc.

Liu, X., D. Duan, W. Li, T. Tadano and M. A. Khan (2006). A Comparative Study on Responses of Growth and Solute Composition in Halophytes Suaeda Salsa and Limonium Bicolor to Salinity. Ecophysiology of High Salinity Tolerant Plants**:** 135-143.

Lonchamp, J. P. (2000). "*Diplotaxis catholica*." Weed Science & Agronomy Retrieved 27 July, 2008, from http://www.dijon.inra.fr/bga/hyppa/hyppa-a/dipca_ah.htm.

Longstreth, D. J. and P. S. Nobel (1979). "Salinity Effects on Leaf Anatomy - Consequences for photosynthesis " Plant Physiology (63): 700-703.

Lynch, J. and A. Laüchly (1985). "Salt stress disturbs the calcium nutrion of barley (Hordeum vulgare L.) New Phytol." (99): 345-354.

Maas, E. V. (1986). "Salt Tolerance of Plants." Applied Agricultural Research(1): 12-26.

Maas, E. V. and G. J. Hoffman (1977). "Crop salt tolerance current assessment." Journal of Irrigation and Drainage Division, ASCE **103**(IR2): 115-134.

Maggio, A., M. P. Reddy and R. J. Jolly (2000). "Leaf gas exchange and solute accumulation in the halophyte *Saladora persica* grown at moderate salinity. ." Environmental and Experimental Botany(44): 31-38.

Makkar, H. P. S. (2003). Quantification of Tannins in Tree and Shrub Foliage - A Laboratory Manual. Dordrecht, The Netherlands, Kluwer Academic Publishers

Marcelis, L. F. M., E. Heuvelink and J. Goudriaan (1998). "Modelling biomass production and yield of horticultural crops: a review." Scientia Horticulturae **74**: 83-111.

Marschner, H. (1995). Mineral Nutrition in Higher Plants. London, Academic Press.

McKersie, B. D. and Y. Y. Leshem (1994). Stress and Stress Coping in Cultivated Plants. Dordrecht/ Boston/ London Kluwer Academic Publishers

Min.Agric. (1996). Country report to the FAO international technical conference on plant genetic resources. FAO international technical conference on plant genetic resources. Leipzig.

Miyamoto, S., E. P. Glenn and M. W. Olsen (1996). "Growth, water use and salt uptake of four halophytes irrigated with highly saline water." Journal of Arid Environments **32**(2): 141–159.

Munns, R. (2002). "Comparative physiology of salt and water stress." Plant, Cell & Environment **25**(2): 239-250.

Munns, R., R. A. James and A. Läuchli (2006). "Approaches to increasing the salt tolerance of wheat ad other cereals." Journal of Experimental Botany (57): 1025 - 1043.

Munns, R. and M. Tester (2008). "Mechanisms of Salinity Tolerance." Annual Review of Plant Biology(59): 651-681.

Naczk, M. and F. Shahidi (2004). "Extraction and analysis of phenolics in food " J. Chromatogr. A(1054): 95-111.

Naeini, M. R., A. H. Khoshgoftarmanesh and E. Fallahi (2006). "Partitioning of Chlorine, Sodium, and Potassium and Shoot Growth of Three Pomegranate Cultivars Under Different Levels of Salinity." Journal of Plant Nutrition **29**(10): 1835-1843.

Nedjimi, B., Y. Daoud and M. Touati (2006). "Growth, water relations, proline and ion content of in vitro cultured *Atriplex halimus* subsp. *schweinfurthii* as affected by $CaCl_2$." Communications in Biometry Crop Science **1**(2): 79-89.

Niazi, B. H. (2007). The response of fodderbeet to salinity: Introduction of a non-conventional fodder crop (Fodderbeet) to salt affected lands of Pakistan. Department of Systems Ecology, Institute of Ecological Science, Faculty of Earth and Life Sciences. Amsterdam, Vrije Universiteit **Ph D-Thesis**

OASE, O. F. A. i. S. E. (2005). "Work Program 2006-2010." Retrieved 18-July-2008, 2008, from http://www.imsa.nl/uploads/OASE_work_programme.

OIA, O. o. I. A. (1990). Saline agriculture salt tolerant plants for Developing Countries. Report of a panel of the board on science and technology for international development. N. R. Council. Washington D.C, National Academy Press, Washington D.C.

Oliveira, H., A. Barros and i. p. Conceição Santos . (2008). "Fourier transformation infrared spectroscopy analysis of salt stressed and fungi infected in vitro grapevine plants." Environmental Experimental Botany.

Poorter, H. and E. Garnier (1999). Ecological significance of inherent variation in relative growth rate and its components. Handbook of Functional Plant Ecology. F. I. Pugnaire and F. Valladares. New York, Marcel Dekker, Inc.**:** 81-120.

Porto, E. R., M. C. C. d. Amorim, M. T. Dutra, R. V. Paulino, L. T. d. L. Brito and A. N. B. Matos (2006). "Rendimento da Atriplex nummularia irrigada com efluentes da criação de tilápia em rejeito da dessalinização de água." **10**: 97-103.

Richardson, S. G. and C. M. McKell (1980). "Water relations of Atriplex canescens as affected by salinity and moisture percentage of processed oil shale." Agronomy Journal(72): 946-950.

Rignanese, L. (2008). *Diplotaxis viminea*. diplotaxis_viminea.jpg.

Ruggiero, M. (2007). The Integrated Taxonomic Information System. Catalogue of Life: 2007 Annual Checklist. G. B. I. Facility.

Salama, S., S. Triverdi, M. Busheva, A. Arafa, G. Garab and L. Erdei (1994). "Effects of NaCl salinity on growth cation accumulation chloroplast structure and function in wheat cultivars differing in sal tolerance." Journal of Plant Physiology **144**: 241-247.

Santos, C. and G. Caldeira (1999). "Comparative responses of Helianthus annuus plants and calli exposed to NaCl. Growth rate and osmotic adjustment in intact plants and calli. ." Journal of Plant Physiology **155**: 769-777

Santos, C., A. Campos and H. Azevedo (2001). "In situ and in vitro senescence induced by KCl stress: nutritional imbalance, lipid peroxidation and antioxidant metabolism." Journal of Experimental Botany **52**(355): 351-360

Santos, M. C. L. V. (1998). Estudo de alguns mecanismos de regulação osmótica e da expressão da glutamina sintetase em células de *Helianthus annuus* L. sujeitas a stress salino: Selecção de células tolerantes a NaCl. . Biology Department Aveiro, Aveiro University. **PhD:** 168.

Schimper, A. F. W. (1891). Pflanzengeographie auf physiologischer Grundlage, Jena:Fischer Publication.

Seemann, J. R. and C. Critchley (1985). "Effects of salt stress on the growth,
ion content, stomatal behaviour and photosynthetic capacity of a
salt-sensitive species, Phaseolus vulgaris L." Planta(164): 151–162.

Shaer, H. M. E. (2006). Halophytes as cash crops for animal feeds in arid and semi-arid regions. Switzerland, Birkhäuser Verlag.

Shah, M., M. F. Strong and C. банка (2000). Food in the 21st Century: from science to Sustainable Agriculture, World Bank Publications.

Shannon, M. C. and C. M. Grieve (1999). "Tolerance of vegetable crops to salinity " Sci. Hort.(78): 5-38.

Strogonov, B. P. (1964). Physiological basis of salt tolerance of plants (as affected by various types of salinity). Jerusalem, Akad Nauk SSSR Translated from Russian**:** 73-97.

Taiz, L. and E. Zeiger (2006). Stress Physiology. Plant Physiology, Sinauwer Associates Inc.

Ueda, A., M. Kanechi, Y. Uno and N. Inagaki (2003). "Photosynthetic limitations of a halophyte sea aster (Aster tripolium L) under water stress and NaCl stress." Journal of Plant Research **116**(1): 63-68.

Ungar, I. A. (1991). Ecophysiology of Vascular Halophytes. Boca Raton, CRC Press.

Uniprot (2008). Diplotaxis catholica. diplotaxis_catholica.jpg.

UNPF, U. N. P. F. (2008). from http://www.unfpa.org/swp/2001/.

UTAD, J. B. d. (2008). "Flora Digital de Portugal." Retrieved 27 July, 2008, from http://aguiar.hvr.utad.pt/pt/herbario/cons_reg_fam2.asp?familia=Brassicaceae&ID=1290.

Ventrella, D., P. Santamaria, V. Magnifico, F. Serio, A. De Boni and S. Cordella (1993). "Influenza dell'azoto sull'accumulo dei nitrati in foglie di rucola (Eruca sativa
Miller) allevata a differenti condizioni di temperatura e irradianza." Riv. di Agron. **27**: 621-626.

Wignarajah, K., D. H. Jennings and J. F. Handley (1975). "The Effect of Salinity on Growth of *Phaseolus vulgaris* L. ." Annals of Botany (39): 1029-1038.

WTW, W.-T. W. G.-. (2002). Operating Manual - inoLab pH Level 2

Yensen, N. P. (2006). Halophyte uses for the twenty-first century. Netherlands, Springer Netherlands.

Zhang, J.-Y. and Z.-Y. Wang (2007). Recent advances in molecular breeding of forage crops for improved drought and salt stress tolerance. Advances in Molecular Breeding Towards Salinity and Drought Tolerance. M. A. J. e. al., Springer**:** 797–817.

Zhu, J.-K. (2001). "Plant salt tolerance." Trends in Plant Science **6**(2): 66-71.

Annexes

1 - RGR (Relative Growth Rate)

Tool for classical plant growth analysis v.1.1 — *Help and FAQs*

Dataset: *Test dataset supplied by the authors* — **Date:** *1-Jun-02*

Input

day	mg			mm²
Time	Weights			Leaf Area
	Root	Non-leaf	Leaf	
7	0.04	0	0.22	5
7	0.07	0	0.29	7
7	0.06	0	0.31	7
7	0.06	0	0.38	10
7	0.03	0	0.25	4
7	0.06	0	0.25	5
7	0.05	0	0.25	5
21	0.9	0	18.4	402
21	0.7	0	11.4	230
21	0.9	0	9.7	248
21	0.4	0	9.4	169
21	1.8	0	10.9	351
21	0.5	0	12.4	283
21	1.6	0	15.5	362
21	1.5	0	13.6	258
21	0.7	0	10.3	206
21	0.7	0	7.8	153
21	0.6	0	12.5	297
21	1.3	0	15.1	270
21	1.4	0	25.3	608
21	1.4	0	17.4	301
21	0.9	0	12.3	334
21	1.7	0	13	281
21	0.8	0	9.4	213

Output

day | g | m²

t_1 : day 7 — t_2 : day 21

Mean Relative Growth Rate / day

Rbar	SE	95% CL
0.266751	0.024354	0.050529

Mean Unit Leaf Rate g / m² / day

Ebar	SE	95% CL
13.70648	2.556379	5.303827

Mean Leaf Area Ratio m² / g

Fbar	SE	95% CL
0.019484	0.003723	0.007724

Mean Leaf Weight Fraction g / g (dimensionless)

Pbar	SE	95% CL
0.887535	0.092129	0.191144

Mean Specific Leaf Area m² / g

Qbar	SE	95% CL
0.022089	0.004379	0.009085

Root-Shoot Allometry

Coeffic.	SE	95% CL
0.77444	0.143002	0.298302

Check on assumptions

Indirect Rbar:	0.268715
Indirect % of direct:	100.7

Figure 17 – Spreadsheet tool for classical plant growth analysis used in this work for calculations of RGR, ULR, LAR, LWF and SLA. "The image displays a spreadsheet example with a specimen set of input and output data. This tool was published by (Hunt et al. 2002).

2 – Mean Values for growth parameters and ionic content

Table 11 – Effect of salinity level on the Initial harvest Biomass. Same letters within each series indicate no significant difference (p>0,05) according to Tuckey's multiple range test. Values are shown with s.e. of means (n=5).

NaCl concentration, mM	Initial Biomass DW (g)
0	0,014±0,001a
50	0,015±0,002a
100	0,015±0,001a
200	0,016±0,001a
300	0,016±0,002a

Table 12 - Effect of salinity level on Stem DW, Final Biomass DW, Leaf DW, Root DW, Leaf Surface and Thickness. Same letters within each series indicate no significant difference (p< 0,05) according to Tuckey's multiple range test. Values are shown with s.e. of means (n=5).

NaCl concentration, mM	Stem DW (g)	Final Biomass DW (g)	Leaf DW (g)	Root DW (g)	Leaf Surface (cm^2)	Thickness (µm)
0	0,034±0,005a	1,34±0,09a	1,14±0,11a	0,36±0,02a	273,31±14,31ab	38,21±0,84c
50	0,039±0,004a	1,52±0,13a	1,18±0,14a	0,33±0,03ab	293,45±16,94a	48,66±3,33bc
100	0,020±0,003a	1,60±0,14a	1,30±0,13a	0,26±0,03b	231,01±13,82b	59,03±4,29bd
200	0,020±0,003a	0,51±0,04b	0,46±0,03b	0,12±0,01c	74,94±3,10c	70,35±4,27a
300	0,014±0,003a	0,30±0,02b	0,27±0,02b	0,05±0,01c	31,67±1,67c	78,91±2,50a

Table 13 - Effect of salinity level on the accumulation of cations in the old and young leaves . Same letters within each series indicate no significant difference (p>0,05) according to Tuckey's multiple range test. Values are shown with s.e. of means (n=5).

	Na ($nmol.mg^{-1}$ DW)		K ($nmol.mg^{-1}$DW)		Ca ($nmol.mg^{-1}$DW)		Mg ($nmol.mg^{-1}$DW)	
NaCl concentration, mM	Young	Old	Young	Old	Young	Old	Young	Old
0	30±7,8a	128±63a	1967±56a	2933±111a	635±39a	209±12b	122±6c	138±6b
50	1575±92b	3409±53b	1200±70c	954±97b	507±36b	158±9bc	98±7c	115±10b
100	2425±59b	4271±270b	980±113c	570±10c	399±15c	102±5bc	99±7c	120±3b
200	4508±256c	6763±504c	337±42b	393±38d	133±14d	39±7c	66±4a	69±8a
300	5000±466c	7720±497c	145±13b	353±55d	40±5d	20±1a	32±3b	49±7a

3 – Statistical analysis

3.1 – Growth

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Initial_Biomass	0	,227	5	,200*	,885	5	,330
	50	,237	5	,200*	,893	5	,371
	100	,313	5	,123	,858	5	,221
	200	,217	5	,200*	,960	5	,810
	300	,307	5	,140	,868	5	,257
Final_Biomass_Log$_{10}$	0	,293	5	,184	,802	5	,084
	50	,312	5	,124	,826	5	,131
	100	,230	5	,200*	,966	5	,848
	200	,225	5	,200*	,925	5	,560
	300	,250	5	,200*	,881	5	,315
Thickness	0	,164	5	,200*	,985	5	,957
	50	,219	5	,200*	,943	5	,684
	100	,264	5	,200*	,869	5	,260
	200	,311	5	,127	,839	5	,162
	300	,283	5	,200*	,858	5	,221
Root_DW	0	,303	5	,149	,854	5	,207
	50	,180	5	,200*	,966	5	,850
	100	,194	5	,200*	,943	5	,687
	200	,358	5	,035	,709	5	,012
	300	,196	5	,200*	,970	5	,876
Stem_DW_Log$_{10}$	0	,243	5	,200*	,891	5	,361
	50	,292	5	,189	,919	5	,521
	100	,326	5	,088	,854	5	,206
	200	,285	5	,200*	,831	5	,141
	300	,390	5	,012	,724	5	,017
Total_leaf_surface_final	0	,202	5	,200*	,945	5	,700
	50	,321	5	,102	,823	5	,123
	100	,268	5	,200*	,843	5	,173
	200	,178	5	,200*	,937	5	,644
	300	,243	5	,200*	,914	5	,489

Total_leaves_DW_Final	0	,343	5	,055	,824	5	,126
	50	,266	5	,200*	,922	5	,545
	100	,291	5	,191	,908	5	,456
	200	,222	5	,200*	,923	5	,553
	300	,157	5	,200*	,980	5	,937
RGR	0	,300	5	,161	,883	5	,322
	50	,259	5	,200*	,841	5	,168
	100	,297	5	,171	,879	5	,307
	200	,287	5	,200*	,906	5	,443
	300	,251	5	,200*	,929	5	,589

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Initial_Biomass	2,178	4	20	,108
Final_Biomass_Log_{10}	1,433	4	20	,260
Thickness	2,305	4	20	,094
Root_DW	2,003	4	20	,133
Stem_DW_Log_{10}	,766	4	20	,560
Total leaf surface final	8,764	4	20	,000
Total_leaves_DW_Final	1,403	4	20	,269
RGR	,864	4	20	,502

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Initial_Biomass	Between Groups	,000	4	,000	,252	,905
	Within Groups	,000	20	,000		
	Total	,000	24			
Final_Biomass_Log_{10}	Between Groups	.382	4	.096	65.829	.000
	Within Groups	,029	20	.001		
	Total	.411	24			
Thickness	Between Groups	5322.954	4	1330.738	24.355	.000
	Within Groups	1092.778	20	54.639		
	Total	6415.732	24			
Root_DW	Between Groups	.346	4	.087	41.703	.000
	Within Groups	.042	20	.002		

	Total	,388	24			
Stem_DW_Log$_{10}$	Between Groups	,000	4	,000	4.830	,007
	Within Groups	,000	20	,000		
	Total	,001	24			
Total_leaf_surface_final	Between Groups	286074.513	4	71518.628	102.862	,000
	Within Groups	13905.798	20	695.290		
	Total	299980.310	24			
Total_leaves_DW_Final	Between Groups	4,406	4	1,102	22.044	,000
	Within Groups	,999	20	,050		
	Total	5,406	24			
RGR	Between Groups	,029	4	,007	49.042	,000
	Within Groups	,003	20	,000		
	Total	,032	24			

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
Initial_Biomass	Welch	,358	4	9,820	,833
	Brown-Forsythe	,252	4	12,558	,903
Final_Biomass_Log$_{10}$	Welch	88.734	4	9.441	.000
	Brown-Forsythe	65.829	4	13.430	.000
Thickness	Welch	60.262	4	8.765	.000
	Brown-Forsythe	24.355	4	14.334	.000
Root_DW	Welch	64.399	4	9.097	.000
	Brown-Forsythe	41.703	4	13.139	.000
Stem_DW_Log$_{10}$	Welch	6.215	4	9.854	.009
	Brown-Forsythe	4.830	4	13.448	.012
Total_leaf_surface_final	Welch	163.996	4	8.853	.000
	Brown-Forsythe	102.862	4	12.015	.000
Total_leaves_DW_Final	Welch	35.071	4	8.956	.000
	Brown-Forsythe	22.044	4	12.070	.000
RGR	Welch	40,933	4	9,867	,000
	Brown-Forsythe	49.042	4	16.288	.000

a. Asymptotically F distributed.

Initial_Biomass

Tukey HSD

Treatment	N	Subset for alpha = 0.05
		1
0	5	,01566
100	5	,01656
50	5	,01698
300	5	,01746
200	5	,01754
Sig.		,905

Means for groups in homogeneous subsets are displayed.

Final_Biomass_Log$_{10}$

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
300	5	,11269	
200	5	,17964	
0	5		,36834
50	5		,39941
100	5		,41184
Sig.		,077	,398

Means for groups in homogeneous subsets are displayed.

Thickness

Tukey HSD

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
0	5	38,20861			
50	5	48,66288	48,66288		
100	5		59,03247	59,03247	
200	5			70,35247	70,35247
300	5				78,91429
Sig.		,207	,214	,150	,384

Means for groups in homogeneous subsets are displayed.

Root_DW

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
300	5	,05400		
200	5	,12120		
100	5		,26420	
50	5		,32560	,32560
0	5			,35760
Sig.		,176	,246	,799

Stem_DW_Log$_{10}$

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
300	5	,0062	
200	5	,0086	,0086
100	5	,0146	,0146
0	5	,0147	,0147
50	5		,0167
Sig.		,056	,077

Means for groups in homogeneous subsets are

Total_leaf_surface_final

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
300	5	31,672000		
200	5	74,944800		
100	5		231,013260	
0	5		273,312000	273,3120
50	5			293,4526
Sig.		,109	,122	,747

Means for groups in homogeneous subsets are displayed.

3.2 – Ions Old Leaves

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Na	0	,306	3		,904	3	,400
	50	,222	3		,985	3	,769
	100	,343	3		,844	3	,223
	200	,180	3		,999	3	,946
	300	,375	3		,775	3	,056
K	0	,275	3	.	,943	3	,541
	50	,185	3	.	,998	3	,925
	100	,377	3	.	,769	3	,043
	200	,220	3	.	,986	3	,776
	300	,319	3	.	,886	3	,341
Mg	0	,361	3	.	,806	3	,130
	50	,358	3	.	,813	3	,145
	100	,260	3	.	,959	3	,609
	200	,314	3	.	,892	3	,362
	300	,256	3	.	,962	3	,623
Ca	0	,322	3	.	,880	3	,326
	50	,381	3	.	,761	3	,024
	100	,321	3	.	,882	3	,332
	200	,282	3	.	,935	3	,508
	300	,385	3	.	,750	3	,000
$Na\_K\_Ratio\_Log_{10}$	0	,308	3	.	,901	3	,390
	50	,176	3	.	1.000	3	,979
	100	,220	3	.	,986	3	,776
	200	,282	3	.	,936	3	,511
	300	,300	3	.	,913	3	,429

a. Lilliefors Significance Correction

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Na	3,290	4	10	,058
K	3,069	4	10	,068
Mg	1,562	4	10	,258
$Ca\ Log_{10}$	6,084	4	10	,010
$Na\_K\_Ratio\_Log_{10}$	3,415	4	10	,052

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Na	Between Groups	1,075E8	4	2,688E7	46,174	,000
	Within Groups	5821004,326	10	582100,433		
	Total	1,133E8	14			
K	Between Groups	4.447E8	4	1.112E8	87.250	.000
	Within Groups	1.274E7	10	1274249.031		
	Total	4.575E8	14			
Mg	Between Groups	17094.198	4	4273.550	15.966	.000
	Within Groups	2676.608	10	267.661		
	Total	19770.806	14			
Ca_Log_{10}	Between Groups	.306	4	.076	26.012	.000
	Within Groups	.029	10	.003		
	Total	.335	14			
$Na\_K\_Ratio\_Log_{10}$	Between Groups	.935	4	.234	83.182	.000
	Within Groups	.028	10	.003		
	Total	.963	14			

Homogeneous Subsets

Na

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
0	3	127,81967		
50	3		3409,38367	
100	3		4270,58833	
200	3			6763,24667
300	3			7719,60800
Sig.		1,000	,651	,565

Means for groups in homogeneous subsets are displayed.

K

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
300	3	2078,		
200	3	2300,		
100	3	3350,	3350,98033	
50	3		5640,87300	
0	3			16584,36867
Sig.		,652	,170	1,000

Means for groups in homogeneous subsets are displayed.

Mg

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
300	3	48,52933	
200	3	69,16200	
50	3		115,15067
100	3		120,29400
0	3		138,35300
Sig.		,560	,456

Means for groups in homogeneous subsets are

Tukey HSD

Ca_Log_{10}

Treatment	N	Subset for alpha = 0.05		
		1	2	3
0	3	1,719		
50	3	1,735	1,73583	
100	3	1,766	1,76629	
200	3		1,88086	
300	3			2,1035
Sig.		,823	,051	1,000

Means for groups in homogeneous subsets

Na_K_Ratio_Log10

Tukey HSD

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
0	5	,0069			
50	5		,364		
100	5		,551		
200	5			1,1730	
300	5				1,5496
Sig.		1,000	,117	1,000	1,000

Means for groups in homogeneous subsets are displayed.

3.3 - Ions Young Leaves

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Na	0	,132	5	,200*	,998	5	,999
	50	,171	5	,200*	,991	5	,984
	100	,136	5	,200*	,990	5	,979
	200	,224	5	,200*	,920	5	,533
	300	,201	5	,200*	,917	5	,512
K	0	,264	5	,200*	,857	5	,219
	50	,256	5	,200*	,903	5	,425
	100	,240	5	,200*	,854	5	,208
	200	,329	5	,081	,804	5	,088
	300	,249	5	,200*	,953	5	,755
Ca	0	,283	5	,200*	,863	5	,239
	50	,239	5	,200*	,882	5	,319
	100	,232	5	,200*	,965	5	,843
	200	,110	5	,200*	,999	5	1,000
	300	,237	5	,200*	,921	5	,536
Mg	0	,264	5	,200*	,814	5	,106
	50	,325	5	,090	,841	5	,167
	100	,298	5	,169	,844	5	,178

	200	,229	5	,200*	,938	5	,649
	300	,184	5	,200*	,951	5	,746
Nitogen	0	,254	5	,200*	,842	5	,170
	50	,204	5	,200*	,921	5	,537
	100	,302	5	,152	,812	5	,102
	200	,212	5	,200*	,940	5	,668
	300	,243	5	,200*	,941	5	,675
Na_K_Ratio_Log	0	,238	5	,200*	,960	5	,809
	50	,175	5	,200*	,978	5	,922
	100	,241	5	,200*	,882	5	,319
	200	,295	5	,180	,840	5	,165
	300	,191	5	,200*	,978	5	,922

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Na	6,378	4	20	,002
K	5,948	4	20	,003
Ca	3,581	4	20	,023
Mg	1,539	4	20	,229
Nitogen	2,050	4	20	,126
Na K Ratio Log	6,303	4	20	,002

ANOVA

		Sum of Squares	df	Mean	F	Sig.
Na	Between Groups	8,514E7	4	2,129E7	72,152	,000
	Within Groups	5900349,965	20	295017,498		
	Total	9,105E7	24			
K	Between Groups	1.060E7	4	2648781.582	115.050	.000
	Within Groups	460456.788	20	23022.839		
	Total	1.106E7	24			
Ca	Between Groups	1258622.166	4	314655.542	96.078	.000
	Within Groups	65499.895	20	3274.995		

	Total	1324122.061	24			
Mg	Between Groups	24585.500	4	6146.375	36.267	.000
	Within Groups	3389.506	20	169.475		
	Total	27975.006	24			
Nitogen	Between Groups	3299.828	4	824.957	24.921	.000
	Within Groups	662.071	20	33.104		
	Total	3961.899	24			
Na_K_Ratio_Log	Between Groups	7,781	4	1,945	146,734	,000
	Within Groups	,265	20	,013		
	Total	8,046	24			

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
Na	Welch	461,583	4	8,101	,000
	Brown-Forsythe	72,152	4	6,753	,000
K	Welch	245.403	4	8.628	.000
	Brown-Forsythe	115.050	4	10.426	.000
Ca	Welch	175.733	4	8.701	.000
	Brown-Forsythe	96.078	4	10.404	.000
Mg	Welch	50.969	4	9.635	.000
	Brown-Forsythe	36.267	4	15.942	.000
Nitogen	Welch	66.756	4	9.327	.000
	Brown-Forsythe	24.921	4	9.446	.000
Na_K_Ratio_Log	Welch	259.981	4	8.086	.000
	Brown-Forsythe	146.734	4	10.231	.000

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-	Std. Error	Sig.	95% Confidence	
						Lower	Upper
Na	0	50	-1,544301E3*	343,521468	,002	-516,35608	
		100	-2,395038E3*	343,521468	,000	-1367,09308	
		200	-4,477761E3*	343,521468	,000	-3449,81608	
		300	-4,969901E3*	343,521468	,000	-3941,95568	
	50	0	1544,301000*	343,521468	,002	516,35608	2572,24592
		100	-850,737000	343,521468	,136	-177,20792	

		200	-2,933460E3*	343,521468	,000	-	-1905,51508
		300	-3,425600E3*	343,521468	,000	-	-2397,65468
	100	0	2395,038000*	343,521468	,000	1367,09308	3422,98292
		50	850,737000	343,521468	,136	-177,20792	1878,68192
		200	-2,082723E3*	343,521468	,000	-	-1054,77808
		300	-2,574863E3*	343,521468	,000	-	-1546,91768
	200	0	4477,761000*	343,521468	,000	3449,81608	5505,70592
		50	2933,460000*	343,521468	,000	1905,51508	3961,40492
		100	2082,723000*	343,521468	,000	1054,77808	3110,66792
		300	-492,139600	343,521468	,615	-	535,80532
	300	0	4969,900600*	343,521468	,000	3941,95568	5997,84552
		50	3425,599600*	343,521468	,000	2397,65468	4453,54452
		100	2574,862600*	343,521468	,000	1546,91768	3602,80752
		200	492,139600	343,521468	,615	-535,80532	1520,08452
K	0	50	767.568400*	95.964242	.000	480.40744	1054.72936
		100	986.763000*	95.964242	.000	699.60204	1273.92396
		200	1630.429600*	95.964242	.000	1343.26864	1917.59056
		300	1822.160200*	95.964242	.000	1534.99924	2109.32116
	50	0	-767.568400*	95.964242	.000	-	-480.40744
		100	219.194600	95.964242	.191	-67.96636	506.35556
		200	862.861200*	95.964242	.000	575.70024	1150.02216
		300	1054.591800*	95.964242	.000	767.43084	1341.75276
	100	0	-986.763000*	95.964242	.000	-	-699.60204
		50	-219.194600	95.964242	.191	-506.35556	67.96636
		200	643.666600*	95.964242	.000	356.50564	930.82756
		300	835.397200*	95.964242	.000	548.23624	1122.55816
	200	0	-1.630430E3*	95.964242	.000	-	-1343.26864
		50	-862.861200*	95.964242	.000	-	-575.70024
		100	-643.666600*	95.964242	.000	-930.82756	-356.50564
		300	191.730600	95.964242	.303	-95.43036	478.89156
	300	0	-1.822160E3*	95.964242	.000	-	-1534.99924
		50	-1.054592E3*	95.964242	.000	-	-767.43084
		100	-835.397200*	95.964242	.000	-	-548.23624
		200	-191.730600	95.964242	.303	-478.89156	95.43036

Ca	0	50	128.106200*	36.193893	.016	19.80051	236.41189
		100	236,516200*	36,193893	,000	128,21051	344,82189
		200	502.797200*	36.193893	.000	394.49151	611.10289
		300	595.458400*	36.193893	.000	487.15271	703.76409
	50	0	-128.106200*	36.193893	.016	-236.41189	-19.80051
		100	108.410000*	36.193893	.050	.10431	216.71569
		200	374.691000*	36.193893	.000	266.38531	482.99669
		300	467.352200*	36.193893	.000	359.04651	575.65789
	100	0	-236.516200*	36.193893	.000	-344.82189	-128.21051
		50	-108.410000*	36.193893	.050	-216.71569	-.10431
		200	266.281000*	36.193893	.000	157.97531	374.58669
		300	358.942200*	36.193893	.000	250.63651	467.24789
	200	0	-502.797200*	36.193893	.000	-611.10289	-394.49151
		50	-374.691000*	36.193893	.000	-482.99669	-266.38531
		100	-266.281000*	36.193893	.000	-374.58669	-157.97531
		300	92.661200	36.193893	.117	-15.64449	200.96689
	300	0	-595.458400*	36.193893	.000	-703.76409	-487.15271
		50	-467.352200*	36.193893	.000	-575.65789	-359.04651
		100	-358.942200*	36.193893	.000	-467.24789	-250.63651
		200	-92.661200	36.193893	.117	-200.96689	15.64449
Mg	0	50	23.537000	8.233475	.066	-1.10064	48.17464
		100	22.789400	8.233475	.078	-1.84824	47.42704
		200	56.112400*	8.233475	.000	31.47476	80.75004
		300	90.016400*	8.233475	.000	65.37876	114.65404
	50	0	-23.537000	8.233475	.066	-48.17464	1.10064
		100	-.747600	8.233475	1.000	-25.38524	23.89004
		200	32.575400*	8.233475	.006	7.93776	57.21304
		300	66.479400*	8.233475	.000	41.84176	91.11704
	100	0	-22.789400	8.233475	.078	-47.42704	1.84824
		50	.747600	8.233475	1.000	-23.89004	25.38524
		200	33.323000*	8.233475	.005	8.68536	57.96064
		300	67.227000*	8.233475	.000	42.58936	91.86464
	200	0	-56.112400*	8.233475	.000	-80.75004	-31.47476
		50	-32.575400*	8.233475	.006	-57.21304	-7.93776
		100	-33.323000*	8.233475	.005	-57.96064	-8.68536
		300	33.904000*	8.233475	.004	9.26636	58.54164

	300	0	-90.016400*	8.233475	.000	-114.65404	-65.37876
		50	-66.479400*	8.233475	.000	-91.11704	-41.84176
		100	-67.227000*	8.233475	.000	-91.86464	-42.58936
		200	-33.904000*	8.233475	.004	-58.54164	-9.26636
Nitogen	0	50	7.908000	3.638875	.230	-2.98088	18.79688
		100	13.072000*	3.638875	.014	2.18312	23.96088
		200	17.010000*	3.638875	.001	6.12112	27.89888
		300	34.448000*	3.638875	.000	23.55912	45.33688
	50	0	-7,908000	3,638875	,230	-18,79688	2,98088
		100	5.164000	3.638875	.623	-5.72488	16.05288
		200	9.102000	3.638875	.130	-1.78688	19.99088
		300	26.540000*	3.638875	.000	15.65112	37.42888
	100	0	-13.072000*	3.638875	.014	-23.96088	-2.18312
		50	-5.164000	3.638875	.623	-16.05288	5.72488
		200	3.938000	3.638875	.813	-6.95088	14.82688
		300	21.376000*	3.638875	.000	10.48712	32.26488
	200	0	-17.010000*	3.638875	.001	-27.89888	-6.12112
		50	-9.102000	3.638875	.130	-19.99088	1.78688
		100	-3.938000	3.638875	.813	-14.82688	6.95088
		300	17.438000*	3.638875	.001	6.54912	28.32688
	300	0	-34.448000*	3.638875	.000	-45.33688	-23.55912
		50	-26.540000*	3.638875	.000	-37.42888	-15.65112
		100	-21.376000*	3.638875	.000	-32.26488	-10.48712
		200	-17.438000*	3.638875	.001	-28.32688	-6.54912
Na_K_Ratio_Log	0	50	-.35808*	.07282	.001	-.5760	-.1402
		100	-.54455*	.07282	.000	-.7625	-.3267
		200	-1.16617*	.07282	.000	-1.3841	-.9483
		300	-1.54276*	.07282	.000	-1.7607	-1.3249
	50	0	.35808*	.07282	.001	.1402	.5760
		100	-.18648	.07282	.117	-.4044	.0314
		200	-.80809*	.07282	.000	-1.0260	-.5902
		300	-1.18468*	.07282	.000	-1.4026	-.9668
	100	0	.54455*	.07282	.000	.3267	.7625
		50	.18648	.07282	.117	-.0314	.4044
		200	-.62161*	.07282	.000	-.8395	-.4037

		300	-.99820*	.07282	.000	-1.2161	-.7803
	200	0	1.16617*	.07282	.000	.9483	1.3841
		50	.80809*	.07282	.000	.5902	1.0260
		100	.62161*	.07282	.000	.4037	.8395
		300	-.37659*	.07282	.000	-.5945	-.1587
	300	0	1.54276*	.07282	.000	1.3249	1.7607
		50	1.18468*	.07282	.000	.9668	1.4026
		100	.99820*	.07282	.000	.7803	1.2161
		200	.37659*	.07282	.000	.1587	.5945

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Na

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
0	5	30,32480		
50	5		1574,62580	
100	5		2425,36280	
200	5			4508,08580
300	5			5000,22540
Sig.		1,000	,136	,615

Means for groups in homogeneous subsets are displayed.

K

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
300	5	144,95120		
200	5	336,68180		
100	5		980,34840	
50	5		1199,54300	
0	5			1967,11140
Sig.		,303	,191	1,000

Ca

Tukey HSD

Treat	N	Subset for alpha = 0.05

ment		1	2	3	4
300	5	39,92380			
200	5	132,58500			
100	5		398,86600		
50	5			507,27600	
0	5				635,38220
Sig.		,117	1,000	1,000	1,000

Mg

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
300	5	31,74080		
200	5		65,64480	
50	5			98,22020
100	5			98,96780
0	5			121,75720
Sig.		1,000	1,000	,066

Nitrogen

Tukey HSD

Treat ment	N	Subset for alpha = 0.05		
		1	2	3
300	5	36,53600		
200	5		53,97400	
100	5		57,91200	
50	5		63,07600	63,07600
0	5			70,98400
Sig.		1,000	,130	,230

Na K Ratio Log_{10}

Tukey HSD

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
0	5	,0069			
50	5		,3649		
100	5		,5514		

200	5			1,1730	
300	5				1,5496
Sig.		1,000	,117	1,000	1,000